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A SIMPLE CULTURE MEDIUM FOR GENERAL USE WITHOUT MEAT EXTRACT OR PEPTONE.

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IN this paper an account is given of a culture medium which is suitable for routine bacteriological purposes and dispenses with the use of meat extract or peptone. Several advantages are claimed :

(1) Its cost of production is small.

(2) It contains no ingredient of which there is likely to be a shortage of supply.

(3) With few or, in some cases, no modifications it is capable of growing all kinds of bacteria met with in routine medical bacteriology. For the most part growth is better than on media containing meat extract and peptone. Good specific staining reactions, e.g. that of Neisser for *C. diphtheriae*, can be obtained.

(4) Once the basal stock solutions, which keep indefinitely, have been prepared, the final medium is simple and rapid to make. Uniformity in different batches is more easily obtained.

The necessary amino-nitrogen content of the medium is supplied by an acid hydrolysate of casein. Since acid hydrolysis partially destroys certain amino-acids, e.g. tryptophan, the hydrolysate is supplemented with a small quantity of tryptic digest of casein. Watery extract of yeast and glutamine† are added to provide most of the essential co-factors. The medium is buffered with sodium glycerophosphate,‡ which may also act as a source of energy.

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† Glutamine is necessary for the growth of certain strains of *Streptococcus haemolyticus* (McIlwain, Fildes, Gladstone and Knight, 1939) and stimulates the growth of some other bacteria (Fildes and Gladstone, 1939). This necessity for glutamine, however, depends largely upon the size of the inoculum. Growth of sensitive bacteria often takes place without added glutamine with the heavy inoculations usual in routine medical work.

‡ This was used in preference to phosphate, since the latter forms an insoluble salt with the calcium in agar media and is almost completely precipitated on autoclaving at pH 7·6. We are indebted to Dr. G. M. Richardson for suggesting the use of glycerophosphate, the calcium salt of which is soluble to 2 per cent.

Sodium lactate is added as a further source of energy. Other additions, e.g. blood and serum, may also be made, depending on the purpose for which the medium is required.

In the first part of the paper exact details are given for the preparation of the medium. In the second part, a description is given of its use in the cultivation of bacteria.

I. TECHNICAL DETAILS.

Apparatus Required.

The following apparatus, which may not ordinarily be available in a bacteriological laboratory, is required: (1) Conical beakers, 1-litre capacity; (2) filter flasks, 2-litre capacity; (3) Buchner filter funnels, 5½ in. diam.; (4) water pump; (5) filter pulp*; (6) Winchester quart bottles; (7) screw-capped bottles ("medicine flats"), 8 oz. capacity, with rubber washers (McCartney, 1933); (8) glass-stoppered reagent bottles, 250 ml. capacity, for storing serum, etc.; (9) tin containers, 6 × 8 in. with "press-down" lids, for incubating cultures in an atmosphere containing CO_2.

Preparation of Stock Substances and Solutions for the Basal Medium.

(A) *Preparation of stock hydrolysed casein.*

This is similar to that used by McIlwain (1939):

To 200 g. of commercial casein† (e.g. Hopkin and Williams' casein commercial, in 3 kg. lots) in a litre conical beaker, add a mixture of 170 ml. concentrated HCl with 110 ml. distilled water. Stir quickly with a glass rod to obtain a uniform suspension before the casein swells and becomes solid. Autoclave at 120° for ¾ hour. Cool, add 40 per cent. NaOH till neutral (about 180 ml.); cool again, and filter through pulp on a Buchner funnel. Dilute to 1 litre, place in a Winchester quart bottle and add 1 per cent. chloroform. Shake vigorously immediately and at intervals to emulsify the chloroform. Store in the dark. One litre of this solution makes about 30 litres of complete medium.

(B) *Stock tryptic digest of casein.*

This is made by a modification of Cole and Onslow's method (1916), which has been used as a routine in the Department of Biochemistry, Cambridge. Put 200 g. of casein ("light white") and 20 g. sodium carbonate crystals in a Winchester quart bottle. Add 2 litres of warm (37°) tap water in two lots of 1 litre each, shaking well after each addition. Add 100 ml. of a suspension of minced pancreas‡ and 10 ml. chloroform, shake well and test pH, which

* The pulp referred to here and elsewhere may be T. B. Ford's 4 per cent. asbestos-cotton filter pulp, sold in slabs. For use, 5 g. are thoroughly whipped up with 500 ml. of distilled water. This suspension is thrown on to a 5½ in. Buchner funnel already protected with a No. 1 Whatman filter paper, and allowed to drain by gravity. It is then sucked dry, and covered with another No. 1 filter paper.

† For mass production the quantity of casein in one beaker should not exceed this.

‡ This is made as follows: Cut off the fat from 7 lb. pig's pancreas, mince finely and suspend in three times the weight of water. Shake vigorously and add alcohol to 25 per cent. Shake before use to get the whole pulp in suspension.

should be 7·4–7·5. Incubate for 16 days at 37°, shaking each day. Samples should be taken daily to check the pH, which should be adjusted if necessary.

After removal from the incubator, allow to stand 2 days and filter off the tyrosine crystals, which settle as a white deposit. To 2 litres of the filtrate add 15 ml. of concentrated HCl in 150 ml. of water and steam for 60 min. Cool, filter and adjust the reaction to pH 7·4 (6–7 ml. of 40 per cent. NaOH are required). Place in a Winchester quart bottle, add 1 per cent. chloroform, shake thoroughly immediately and at intervals to keep the chloroform emulsified, and store in the dark.

(C) *Stock extract of yeast.*

Crumble 250 g. pressed brewer's yeast* into 1 litre of distilled water, boiling over a naked flame. Stir continuously until frothing ceases (about 5 min.). Filter on a Buchner filter through pulp. Store in Winchester quart bottles in the dark, after adding 1 per cent. chloroform and shaking vigorously.

For mass production, each boiling may be pooled and left in the cold overnight. The supernatant is then syphoned off, run through pulp and then bottled as above. The deposit may be discarded.

(D) *Sodium glycerophosphate.*

This is kept as the solid salt of commercial grade.

(E) *Glutamine.*

A 0·05 per cent. solution in distilled water is sterilized by filtration and kept in the ice-chest. Glutamine solutions are not stable indefinitely and small quantities only should be made up. The stock of glutamine crystals should be preserved in a desiccator in the ice-chest. Glutamine is used in a final concentration of 0·00025 per cent.; one g., therefore, will be sufficient for 400 litres of medium. Glutamine is obtainable from the British Drug Houses, Ltd.

(F) *Sodium lactate.*

Commercial sodium lactate may be bought as a 50 per cent. syrupy solution.

Substances and Solutions Required to be Added to the Basal Medium for Special Purposes.

The solutions and substances described above are for the preparation of the basal medium. For special purposes, described later, one or more of the following must be added :

(G) *Glucose.*

For stock a 25 per cent. solution in distilled water is kept in suitable bulk after sterilization in the autoclave.

* Many brewers will supply yeast free.

(H) *Lactose.*

This is kept as a 20 per cent. solution, also autoclaved. No appreciable hydrolysis occurs on autoclaving a 20 per cent. solution.

(I) *Litmus solution.*

This is that of McIntosh (1920) containing 40 per cent. alcohol. It has been found in practice an advantage to remove the alcohol by distillation and to make up the volume with distilled water, when the solution can be sterilized by autoclaving and added to sterile basal medium in bulk. This obviates the necessity for steaming on three successive days.

(J) *Serum.**

Horse serum may be prepared by the method described by Fildes and McIntosh (1931) or by any other method.

(K) *Laked ox blood.**

This is that of Fildes and McIntosh (1931). It is used for enrichment in place of horse serum where this is difficult to obtain. It may be used either unheated or for the preparation of " chocolate " agar.

For haemolysis tests, whole blood from a rabbit or other source is added as with ordinary laboratory media.

(L) *Fildes' peptic digest of sheep blood.*

This is made in the same way as that described by Fildes (1920), except that digestion is carried out at 37° for 3 days, as recommended by Glass (1939), and the reaction is adjusted to pH 6·8 initially, without first bringing the pH to 7·6.

(M) *Cystine solution.*

A 0·12 per cent. solution in $N/10$ HCl is sterilized by filtration and kept in the ice-chest.

Other additions.

Other additions, e.g. sugars for fermentation tests, are added as with ordinary laboratory media.

Preparation of the Basal Casein-Casein-Yeast Medium (CCY).

Solid medium.

Whip up 15 g. powdered agar in 850 ml. distilled water, adding the water gradually to make a smooth paste and finally a uniform suspension. Place

* It should be noted that the successful collection of serum or blood suitable for media at a slaughter-house depends largely on the skill with which the blood is directed from the jugular vein into the vessel without gross contamination. Most of the contaminating bacteria in the Fildes-McIntosh (1931) method are removed by the clot produced in the plasma or laked blood. Gross contamination cannot be overcome by the chloroform subsequently added.

in the autoclave, already hot, and allow to steam for 5 min., then run up to 1 atmosphere and turn off heat. Open in half an hour.

Take—

Casein (A)	35 ml.
Casein (B)	15 ,,
Yeast (C)	100 ,,
Sodium glycerophosphate (D)	10 g.
Sodium lactate (F)	10 ml.

Shake to dissolve the glycerophosphate, heat the mixture to 100° and mix with the hot (100°) agar solution. Add 3·6 ml. of 40 per cent. NaOH (to increase the pH to about 9·3),* place in the steamer and test the temperature, which should not be less than 90–95°. At this point a precipitate forms and is allowed to deposit. Filter *at once* as follows: Prepare a Buchner funnel with pulp as already described (footnote, p. 162); fit it to a filter flask attached to a water pump and stand in the steamer until the funnel is very hot. Turn on the pump and pour the hot agar on to the upper filter paper. To avoid prolonged heating at a high pH, filtration should be as rapid as possible. For this purpose, the bulk of the precipitate, which soon settles to the bottom, should not be shaken up. The supernatant passes through the filter rapidly (about 5–10 min.) and can be removed from the steamer. The small amount of residue may then be passed through the filter or may be discarded. The filtered agar must not be allowed to stand at this high pH; therefore, without delay, add 2·05 ml. concentrated HCl (sp. gr. 1·142, 32 per cent.) per litre. If more than one litre of medium is being made, the NaOH should only be added to one litre at a time and this filtered and the pH readjusted, before another litre is treated in the same way. Meanwhile the rest of the medium before the addition of NaOH is kept hot in the steamer. Not more than 1 litre should be passed through each filter. Finally pool the filtered agar and check the pH,† which should be 7·6, using cresol red as the indicator. Add:

Glutamine solution‡ (E)	5 ml. per litre.

Distribute in tubes or bottles and autoclave at 115° for 20 min.

Liquid medium.

The preparation of the liquid medium is similar to that of agar, except that the agar solution is replaced by 850 ml. of distilled water. In the absence of

* This procedure is rendered necessary by the presence of free phosphate in commercial supplies of sodium glycerophosphate. This combines with the calcium of the agar. When heated at an alkaline pH (about 9·3) the calcium phosphate is precipitated and can be filtered off. This is similar to the well-known method of removing phosphate in the preparation of nutrient meat broth.

† If no standard tints are available, the following method can be used: Make up and keep in the ice-chest $M/10$ solutions of KH_2PO_4 (1·36 per cent.) and Na_2HPO_4 12 H_2O (3·58 per cent.). Make up 0·04 per cent. solution of cresol red in 50 per cent. alcohol. Arrange the comparator in the usual way. The standard tint (pH 7·6) is made by mixing 0·9 ml. of the KH_2PO_4 solution with 9·1 ml. of the Na_2HPO_4 solution and 0·3 ml. of the cresol red. The same amount of indicator is added to 10 ml. of the medium. The standard tube should be warmed to the same temperature as the agar.

‡ Glutamine, although rapidly destroyed by heat in watery solution, appears to be stable to autoclaving in CCY agar.

agar, calcium is not present in a sufficient concentration to produce a precipitate with the phosphate present in the medium. There is, therefore, no necessity to heat the medium at an alkaline pH during the preparation.

This medium, whether solid or liquid, is a suitable substitute for meat infusion agar and broth for general purposes. It will support the growth of all bacteria of medical interest which have been tested and are able to grow on meat media.

Modifications for Special Purposes.

Serum medium.

For organisms requiring serum for growth, the medium is supplemented with 5 per cent. horse serum (J) after sterilization.

As described later, this medium is also a suitable substitute for Loeffler's medium for *C. diphtheriae*. For this purpose it is important to use horse serum ; rabbit, sheep and human sera are unsuitable.

Blood medium.

Whole blood may be added to CCY medium for haemolysis tests. Laked ox blood (K) may be used in place of serum for enrichment (except as a substitute for Loeffler's medium for *C. diphtheriae*) as described above. It is added in a concentration of 10 per cent.

A modified Fleming's (1919) " chocolate " agar (e.g. for gonococcus, etc.) may be made as follows : 10 per cent. laked ox blood is added to sterile bottles of CCY medium. If required for plates, the bottles are boiled or steamed for 5 min. and plates poured ; if for tubes, the medium is tubed before boiling, the tubes sloped and steamed in the sloped position. Since there is a higher percentage of blood than that normally present in " chocolate " agar, the precipitate tends to separate leaving a clear medium on the surface. This, however, is unimportant.

Influenza medium.

Fildes' peptic sheep blood digest (L) is added in a concentration of 5 per cent. to sterile melted CCY agar cooled to 50°, or to CCY broth.

Litmus lactose agar.

For the growth of organisms of the enteric group, the medium need not contain more than 1/10 of the quantity of yeast present in CCY. For the sake of economy, therefore, all media required exclusively for the growth of organisms of this group, e.g. litmus lactose agar, should only contain 10 ml. yeast extract per litre. Glycerophosphate is omitted.*

To make CCY litmus lactose agar, the following solutions are added to 1 litre of CCY agar containing the reduced quantity of yeast and with glycerophosphate omitted :

Lactose solution (H)	50 ml.
Litmus solution (I)	35 ,,

* In the preparation of basal agar for this medium there is no necessity to bring the pH to 9·3 before filtration. The medium should be adjusted to pH 7·6 and the calcium phosphate then filtered off. No more is precipitated on autoclaving.

The medium is distributed in 12–20 ml. lots in tubes for pouring into plates and is sterilized by steaming on three successive days.

It will save time to add sterile solutions to a sterile basal medium. Alcohol is removed from the litmus solution as described above, and both litmus and lactose solutions autoclaved separately. The requisite quantities are then added to bottles of sterile melted CCY agar, and the medium either stored in bottles or distributed in tubes for pouring into plates.

A modified MacConkey medium can also be made with CCY agar.

Glucose medium.

The growth of *Streptococcus haemolyticus* and pneumococcus is very markedly improved by the presence of glucose. Ten ml. of glucose solution (G) are added per litre either before or after the medium is autoclaved. For the pneumococcus, serum must also be present. Glucose should not be added to blood plates for haemolysis tests, as a greenish-brown discoloration of the medium is produced during growth, which obscures the zone of haemolysis round the colonies.

Diphtheria differential medium.

For differentiating between *gravis*, *mitis* and *intermedius* types of *C. diphtheriae*, a suitable medium is as follows :

To 1 litre of melted CCY agar add :

Peptic sheep blood (L)	50 ml.
Cystine solution (M)	5 ,,
N NaOH	0·5 ,,

Mix thoroughly and autoclave at 115° for 20 min. As described later, autoclaving is necessary and must not be omitted.

Media for indole production and fermentation tests (enteric organisms).

As described later, CCY broth with 1/10 of the quantity of yeast normally present will replace peptone water for growing enteric organisms for indole production tests. If glycerophosphate is omitted, it is a good basal medium to which sugars may be added for fermentation tests.

Carbon dioxide.

Although it has long been recognized that carbon dioxide is an important factor for bacterial growth, little practical use has been made of this knowledge, except in the isolation of certain organisms. Auger (1939) emphasizes the importance of using carbon dioxide in growing pneumococci and other organisms from sputum. An instance of this has been noted by Dr. Gleeson White in this Institute, who found that pneumococci from sputum from a case of pneumonia failed completely to grow on nutrient broth blood agar in air, whereas the same cultures placed in 5 per cent. CO_2 grew copiously. Such instances strongly suggest the advisability of the general use of CO_2 for cultures for diagnostic purposes.

The use of carbon dioxide is particularly important for bacterial growth

on CCY media. This is especially true of surface cultures on solid medium. Such cultures should always be incubated in an atmosphere containing 5 per cent. CO_2.

A simple method of growing bacteria in 5 per cent. CO_2 is to use tin containers (6 × 8 in.) of capacity 3600 ml., with "press-down" lids.* CO_2 is generated in the tin from marble and HCl. The cultures, either plates or tubes, are inserted together with an open tube containing 8 ml. (excess) of 25 per cent. HCl. A marble chip of about 0·7 g. weight (calculated to give approximately 5 per cent. concentration of CO_2 at 37°; since the exact concentration is unimportant, the weight of the marble need only be very approximate) is dropped into the acid and the lid pressed down. The increased pressure due to the CO_2 is not important. Further cultures may be added without using fresh marble and acid, provided this is done reasonably quickly and without disturbing the internal atmosphere when removing the lid.

During incubation, moisture tends to collect in the lid of the plate. It is, therefore, advisable before incubation to place in the lid of the plate a square piece of sterile filter or blotting paper of such a size that it is just held in position by its four corners, e.g. for a 4-in. plate, 3 in. square paper. The paper should not fill the top of the plate, as it tends then, when wet, to prevent the gaseous interchange between the air in the plate and that outside.

II. GROWTH OF BACTERIA ON CCY MEDIUM.

Table I shows the medium of choice for growing each of the more common bacteria met with in routine medical work.

Some account of the growth of these bacteria on CCY medium and its modifications is given below.

Streptococcus.

Good growth of *Streptococcus haemolyticus* is obtained on CCY agar without other addition. Growth in most cases was heavier than on nutrient broth agar. Strains tested included 10 Group A, 2 Group B, 2 Group C, 2 Group G and many others not grouped. Primary isolation of streptococci from pus, etc., was satisfactory. *Streptococcus viridans* also grew well on CCY agar.

Serum improves growth to some extent, but the most marked improvement is obtained on the glucose medium. On this medium the colonies were large, irregular and in many cases mucoid, resembling *Streptococcus mucosus*.

Growth in liquid CCY medium was also good; with glucose present a very massive granular growth was obtained.

Viable counts of a suspension of the "Richards" strain (Group A) of haemolytic streptococcus, using the Wilson roll tube technique, were made on CCY glucose agar and compared with counts of the same suspension on meat broth agar. On CCY glucose agar the count was 101×10^6 per ml.; on meat broth agar it was $92{\cdot}7 \times 10^6$ per ml. The colonies on CCY glucose agar were larger and considerably more easy to count.

CCY blood agar and broth are satisfactory media for haemolysis tests. As stated above, glucose should not be added for this purpose.

* Such tins may be obtained from Messrs. A. Gallenkamp & Co., Ltd., A wire basket for holding plate cultures is useful and can easily be constructed, or may be obtained from this firm.

TABLE I.—*Bacteria Able to Grow on CCY Medium and its Modifications.*

Medium.	Organisms.	Remarks.
CCY alone	*Bact. coli, Bact. typhosum* and other enteric organisms.	..
	Staphylococcus.	..
	(Streptococcus.)*	..
	(*C. diphtheriae.*)	..
	(*Br. abortus.*)	..
	(*Clostridium* group.)	..
	Other organisms normally able to grow on meat-peptone media.	..
CCY + glucose (0·25 per cent.)	Streptococcus.	..
	Clostridium group (except *Cl. tetani.*)	..
CCY + horse serum (5 per cent.)	Meningococcus, gonococcus.	..
	(Pneumococcus.)	..
	Br. abortus.	..
	C. diphtheriae.	Replacing Loeffler's medium.
CCY + serum (5 per cent.) + glucose (0·25 per cent.)	Pneumococcus.	..
CCY + whole blood (5 per cent.)	Streptococcus, pneumococcus, etc.	For haemolysis tests.
	H. pertussis.	
CCY + Fildes' digest (5 per cent.)	*H. influenzae.*	..
	Cl. tetani.	..
Ditto + cystine (autoclaved)	*C. diphtheriae.*	Differential medium.
CCY (no glycerophosphate, reduced quantity of yeast) + litmus + lactose (1 per cent.)	*Bacterium* group.	,, ,,

* Organisms placed in brackets grow better on another medium than that against which they are listed. Growth on the latter, however, is quite adequate for routine diagnostic purposes.

Pneumococcus.

Many strains of pneumococcus grow on CCY medium without other addition. All strains tested (9) grew well on CCY serum or blood agar, and this is the medium recommended for routine use. Glucose considerably improves growth, which is in many cases mucoid in character. Primary isolation, e.g. from sputum, on serum and blood CCY agar was satisfactory.

Staphylococcus.

Staphylococcus grows well on CCY agar. Pigment production by *Staphylococcus aureus* is particularly well marked.

Clostridium *Group.*

Cl. tetani, Cl. welchii, Cl. botulinum, Cl. sporogenes and *Cl. septique* were tested for growth in CCY agar shake and plate cultures. The latter were incubated anaerobically in hydrogen containing 5 per cent. CO_2. The growth of *Cl. welchii, Cl. sporogenes* and *Cl. septique* was good, although on plate cultures it was slightly less than on nutrient broth agar. Marked improvement of growth was obtained, particularly with *Cl. welchii*, when 0·25 per cent.

glucose was present. *Cl. tetani* and *Cl. botulinum* grew rather less well on plate cultures, although shake cultures were comparable to nutrient broth agar. The growth of *Cl. botulinum* was much improved with glucose present. By the addition of peptic sheep blood (5 per cent.) *Cl. tetani* grew well, growth being equal to that on peptic sheep blood broth agar. *Cl. tetani* was successfully isolated from soil on this medium.

From these results it is evident that the best medium for growing most of the members of this group is CCY glucose agar. For slope and plate cultures of *Cl. tetani*, CCY peptic sheep blood agar should be used.

Neisseria *Group.*

Meningococcus.

The meningococcus grows very heavily on CCY agar enriched with 5 per cent. serum or blood. One strain, freshly isolated, produced colonies 3 or 4 times as large as those on nutrient broth agar with 10 per cent. serum. Primary isolation from cerebro-spinal fluid was satisfactory. Neither of two recently isolated strains would grow without serum or blood. After several subcultures, one strain grew slightly after 18 hours; growth was very heavy after 2 days. A third strain (obtained from Dr. V. Glass) grew well without serum or blood. Dr. R. Cruickshank (private communication) succeeded in isolating several strains from cerebro-spinal fluid on CCY agar without added serum. His method of inoculation, however, was to flood the surface of the medium with the fluid. It is probable that growth was the result of enrichment by the fluid in the inoculum.

Gonococcus.

Two strains from conjunctivitis were successfully isolated on CCY medium with 5 per cent. serum. Eighteen other strains from laboratory cultures were also tested. Of these 12 were recently isolated (3 from Prof. A. Fleming, 7 from Dr. I. N. O. Price and 2 from this Institute). The growth of all was equal to that on broth agar containing serum. All required serum or blood for growth. Six other strains (from Dr. I. N. O. Price) were old cultures. These all grew on CCY agar without serum.

Haemophilus *Group.*

H. influenzae.

H. influenzae grows satisfactorily on CCY Fildes' agar, but growth is slightly inferior to that on a medium containing meat and peptone. One strain was tested.

H. pertussis.

One strain from the National Collection of Type Cultures was tested. Satisfactory growth was obtained on CCY medium with 5 per cent. serum or rabbit's blood.

Brucella *Group.*

Two strains of *Br. abortus* (from Prof. G. S. Wilson) were tested. Both grew on CCY medium, but more slowly than on nutrient broth agar. Growth was improved by the addition of serum. These strains showed marked CO_2 sensitivity. Using the technique for growing organisms in an atmosphere of 5 per cent. CO_2 described above, good growth was obtained. This shows that the technique was adequate for the purpose.

Corynebacterium *Group.*

Good growth of *gravis* and *mitis* strains of *C. diphtheriae* was obtained on CCY agar in 18 hours. This became very heavy on further incubation (2 days). The growth of *intermedius* strains, although not so good, was better than on nutrient broth agar. The difference in colony appearance between the three forms was well marked.

The addition of 5 per cent. horse serum markedly increases growth. Very massive growth, increasing up to the second day, is produced. Colony differentiation between the three types is not found with horse serum present, but organisms grown on this medium stain well by Neisser's stain. In this respect it is comparable with Loeffler's medium, and can be used to replace this in diagnostic cultures from throat swabs. It has the added advantage of being transparent. CCY serum agar has not quite the same selectivity for *C. diphtheriae* that Loeffler's medium has ; owing to this, if other organisms are present, diphtheria bacilli in stained films from throat swab CCY serum agar cultures appear to be fewer in number than in films taken from Loeffler cultures. Nevertheless, on comparing this medium with that of Loeffler for routine throat swabs over a period of six months, Dr. C. J. C. Britton (private communication) obtained the same number (15) of positive results on each.

Differentiation between *gravis*, *mitis* and *intermedius* types, although appreciable on CCY medium without other addition, is well marked on the differential medium described above containing peptic sheep blood digest.* Colony differentiation is apparent after 18 hours, but differences are most marked on the second day and growth is then very heavy. As with other differential media, the bacilli do not stain well with Neisser's stain.

It is not suitable to add tellurite to this medium, since a concentration of 0·04 per cent. (recommended by Glass, 1937) is completely inhibitory to growth. If a tellurite medium is required, a modification of that of Anderson, Happold, McLeod and Thomson (1931) can be made, using CCY as the basal medium. Bottles of CCY agar are melted and cooled to 75°. Potassium tellurite (0·04 per cent.) sterilized by filtration and defibrinated rabbit's blood (7–10 per cent.) are added and the medium is then heated at 75° for 10–15 min. and poured into plates.

These observations have been based on a study of 28 laboratory strains,

* Glass (1939) found that peptic sheep blood digest was toxic for the growth of certain *mitis* strains, but that this was diminished by heating the medium. If CCY peptic sheep blood agar is used without autoclaving, this inhibition is very marked with all strains. Autoclaving almost completely destroys this effect. Growth is slightly slower than on the medium without peptic digest. Two " sensitive " *mitis* strains, obtained from Glass, grew as well as other *mitis* strains.

12 *mitis*, 12 *gravis* and 4 *intermedius*. Several were freshly isolated. Twelve were obtained from Dr. V. Glass.

Bacterium *Group*.

Bact. typhosum and other Salmonella organisms, *Bact. shigae*, *Bact. flexneri* and organisms of the *Bact. coli* group, all grow well on CCY litmus lactose agar. Growth of the non-lactose fermenters is slightly less than on nutrient broth litmus lactose agar. Differentiation is well marked. The pH of the medium in plates incubated in 5 per cent. CO_2, a procedure which is very necessary for these organisms growing on this medium, falls slightly (in this medium the buffer is omitted); consequently the medium becomes reddish purple in colour. Differentiation, however, is still easy, and the medium regains its former colour very quickly on standing in air.

CCY broth (containing 10 ml. yeast extract (C) per litre and no glycerophosphate) with sugars and indicator, e.g. phenol red, added is a satisfactory medium for fermentation tests. In liquid media there is less necessity to incubate cultures in an atmosphere containing CO_2, presumably because the CO_2 produced by the organism does not diffuse away so rapidly as on the surface of a solid medium. For fermentation tests it is preferable not to use CO_2 for incubation, since its action on the indicator may be interpreted as slight fermentation by the organism. Moreover, unlike the colour of a solid medium, that of a liquid medium is not readily restored on standing in air. Dr. Gleeson White in this Institute had no difficulty in isolating and identifying *Bact. typhosum* from faeces using these media.

CCY broth with the reduced quantity of yeast* is an excellent medium for detecting indole production by *Bact. coli*.

CCY agar can be used for growing Salmonella organisms in bulk in the preparation of vaccines, etc.

Reports from Other Laboratories.

The medium has been supplied to several laboratories in this country for routine bacteriological diagnosis. The reports, although differing in detail, have been for the most part favourable.

SUMMARY.

An easily prepared culture medium suitable for routine medical bacteriology is described. No meat or peptone is necessary.

We have to thank a number of colleagues who have been able to supply us with reports of their tests during the evolution of the medium, namely, Lt.-Col. J. S. K. Boyd, R.A.M.C., Dr. C. J. C. Britton, Dr. R. Cruickshank, Dr. A. Felix, Prof. A. Fleming, Dr. M. H. Gleeson White, Surg.-Commander F. G. Hitch, R.N., Dr. J. E. McCartney, Prof. J. McIntosh, Dr. A. B. Rosher, Dr. W. M. Scott, Prof. W. C. C. Topley and Prof. H. D. Wright.

* For this purpose it is immaterial whether glycerophosphate is present or not, since it does not appear to affect indole production.

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EXPERIMENTS ON THE GROWTH IN VITRO OF THE VIRUS OF YELLOW FEVER.

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DURING the routine production of yellow fever vaccine a number of experiments have been carried out on the conditions necessary for the growth in vitro of the virus of yellow fever. The ultimate aim of these experiments was to find a medium which would support the growth of the yellow fever virus in the absence of living cells; although this aim has not been fulfilled, certain results of interest have been obtained.

TECHNIQUE.

Unless otherwise stated the strain of yellow fever virus used in these experiments was the attenuated viscerotropic virus 17D between the 200th and 250th passage. Cultures were carried out in 50 c.c. Erlenmeyer flasks, each flask containing 5 c.c. of fluid. The fluid in the control flasks consisted of 1 part of normal human serum, inactivated for 20 minutes at 56° C. and filtered before use through a Seitz EK filter, and 9 parts of Tyrode's solution, the composition of which was as follows: NaCl 8·0 g.; KCl 0·2 g.; $CaCl_2$ 0·2 g.; $MgCl_2$ 0·1 g.; NaH_2PO_4 0·05 g.; $NaHCO_3$ 1·0 g.; glucose 1·0 g.; dissolved in 1000 c.c. of double glass-distilled water. To the serum Tyrode were added

2 drops of finely minced chick embryo, the embryos being from eight to ten days old; this is referred to as the standard medium. As inoculum 1 c.c. of virus-containing material was added to the serum Tyrode mixture. The flasks were incubated at 37° C. and subcultures were made at intervals of four days. The presence of virus was determined by the intracerebral injection of 0·03 c.c. of the supernatant fluid into mice. For titration of the virus decimal dilutions of the original culture fluid were made in 10 per cent. serum saline immediately before the intracerebral injection of mice. The experiments may be divided into those in which changes were made in the cellular content of the standard culture medium, those in which some alteration was made in the fluid portion, and those in which alterations were made both in the cellular and fluid contents. As controls in every case cultures were made in the standard medium.

CHANGES IN THE CELL CONTENT OF THE STANDARD MEDIUM.

When the attenuated strain of yellow fever virus was first grown in vitro either with mouse or chick embryo cells, care was taken to remove the heads and spinal cords of the embryos for fear that growth in nervous tissues would lead to a sudden increase in neurotropism of the cultivated virus.

Theiler and Smith (1937) believed that the presence of whole chick embryonic tissue, thus including nervous tissues in the serum Tyrode medium, did produce some slight increase in the neurotropism of the yellow fever virus. Penna and Moussatche (1939), however, found that after between 19 and 39 passages in the developing embryo in vivo where nervous tissues were inevitably present, both the viscerotropism and neurotropism of a fully virulent strain of viscerotropic virus were greatly reduced. In order to determine whether the neurotropism of 17D was modified by growth in vitro in the presence of a high concentration of embryonic nervous tissue, the following experiment was carried out: Tissue culture virus from the same flask was inoculated into two flasks. The first flask contained chick embryo tissue after removal of the head and spinal cord, while the second flask contained only embryonic brain and spinal cord. Fifty passages of the virus were made in these two media. After the first 20 passages instead of chick embryo the tissues consisted of mouse embryo tissues. After every 5 subcultures in tissue culture the supernatant fluid was inoculated intracerebrally into six mice in amounts of 0·03 c.c.; the length of time between intracerebral inoculation and the development of nervous symptoms in mice was noted. The first mouse to develop symptoms was killed, its brain removed and further inoculated intracerebrally into mice. This process was continued until all the mice developed symptoms on the fourth day after inoculation, regarded as complete neurotropism. When a fully virulent viscerotropic yellow fever virus is thus subinoculated intracerebrally into mice, it usually takes from 9 to 12 intracerebral passages before complete neurotropism has developed. It was considered that if growth in tissue culture containing embryonic nervous tissues enhanced the neurotropism of the virus, the number of mouse intracerebral passages required to produce complete neurotropism would be reduced.

Reference to Table I shows that the strain of virus grown in embryonic nervous tissue was not more neurotropic than the virus grown in the absence of embryonic nervous tissues. Embryonic nervous tissues whether from the chick or from the mouse do not, therefore, appreciably alter the neurotropism of the tissue culture virus grown in vitro, whereas in vivo adult mouse nerve tissues after a few passages cause a marked increase in neurotropism.

TABLE I.—*Neurotropism in the Yellow Fever Tissue Culture Virus 17D.*

Number of tissue culture passages.	Number of mouse brain passages required to produce nervous symptoms on fourth day.	
	Virus grown *in* nervous tissues.	Virus grows *without* nervous tissues.
1	10	12
10	11	10
20	13	11
30	9	10
40	10	12
50	11	9

In order to find whether adult mouse brain tissue would act in vitro in the same way as it does in vivo, an attempt was made to replace the embryonic nerve tissues in the tissue-culture flasks by minced adult mouse brain. Survival of the virus occurred only for one or two subpassages. Since, therefore, under the experimental conditions employed adult mouse brain tissues do not support growth of the virus in vitro, no comparison could be made between the action of adult mouse nervous tissues in vivo and in vitro.

Attempts have been made to replace minced embryo by other tissues and successful growth of the virus has been obtained in human placental tissue, as well as in mouse carcinoma and sarcoma tissue. Full-time human placentas were obtained through the kindness of Prof. F. J. Browne. To obtain sterile placental tissues, the placenta is placed on delivery in a sterile vessel with the membrane side uppermost; the membranes are then seared with a hot iron, divided with sterile instruments and portions of the subjacent tissues removed. These portions of tissue should not be greater than 1 c.c. in volume. Before being placed in the serum Tyrode medium the placental tissue is finely minced in the same way as is done with the mouse or chick embryo tissues. Ten subcultures were made over a period of five weeks in human placental tissues and the titre, 10^{-3}, of the final subculture was equal to that of the original material with which the culture was started. Poleff (1939) has found that human placental tissues are also suitable for the growth in vitro of vaccinia virus.

The tissues of a transplantable mouse sarcoma 37S and of a transplantable mouse carcinoma M63 were also found to give satisfactory growth when used in place of embryo tissues. Seven subcultures were made with 37S and six with M63. Findlay and MacCallum (1937) found that a fully viscerotropic yellow fever virus could grow in a mouse carcinoma in vivo, gradually losing a considerable degree of its viscerotropism without acquiring increased

neurotropism. No change was detected in the pathogenicity of the virus as a result of growth in malignant tissues.

Embryonic chick tissues suspended in Tyrode were heated for 30 minutes at 56° C. before being added to the serum Tyrode medium. No growth of the yellow fever virus occurred in the presence of heated cells. In addition no growth of virus occurred when the living embryonic cells were replaced by finely minced portions of a Seitz EK filter pad. When such portions of filter pad and embryonic cells were both present in the serum Tyrode medium the virus titre was always less than when cells alone were present.

From time to time claims have been made that vaccinia virus would grow in vitro in the presence of yeast cells in place of tissue cells. Two drops of a thick suspension of baker's yeast were added to 4 c.c. of serum Tyrode medium together with 1 c.c. of the yellow fever virus suspension. After incubation for four days the mixture was filtered through a Berkefeld V filtrate before being inoculated intracerebrally into mice; no evidence was obtained of the growth or even survival of the virus in the presence of yeast cells. Similar negative results were obtained when a suspension of *B. coli* was used in place of embryo cells. These few experiments gave negative results, but since certain bacteria and viruses are often associated in vivo, it is possible that bacteria may be found which would allow of the in vitro growth of certain viruses.

An attempt was also made to determine whether larval *Aëdes aegypti* could replace embryo tissue in the growth in vitro of the tissue culture virus. Through the kindness of Mr. Boris Jobling a supply of *Aëdes aegypti* larvae grown under sterile conditions was obtained. After removal from the culture fluid the larvae were washed three or four times in Tyrode's solution and were then minced. The minced tissues of at least six larvae were added to each flask. Five experiments were carried out with the tissue culture virus 17D and five with the neurotropic strain of yellow fever virus, the virus-containing material in the latter case being derived from infected mouse brain after filtration through a Seitz pad. No definite growth of either strain of the virus was obtained when the larval *Aëdes aegypti* tissues were substituted for those of chick or mouse embryo. In two instances with the neurotropic strain and once with 17D the virus survived the second passage. When more favourable circumstances arise it is hoped to carry out similar experiments with a fully virulent strain of yellow fever virus. The negative results so far obtained are of interest since they are consonant with the numerous in vivo experiments, which show that it is not easy to infect adult *Aëdes aegypti* with either the neurotropic or the 17D strain of yellow fever virus.

No growth of the 17D virus was obtained in the serum Tyrode medium in the total absence of animal cells.

VARIATION IN THE FLUID CONSTITUENTS OF THE TISSUE CULTURE MEDIUM.

In addition to changes in the cellular contents of the standard medium, a number of experiments were carried out in which variations were introduced in the fluid portion of the medium.

The first change attempted was to determine whether serum was necessary for the growth of the virus. A medium without serum consisting of Tyrode's solution and chick embryo tissue was therefore inoculated with the tissue culture virus. It was found that growth occurred just as readily in the absence as in the presence of serum. Thirty-five passages were made in the absence of serum, and titration of the virus concentration on a number of occasions showed that the concentration of virus was equal to that attained in the presence of serum.

The next change was to determine whether in the presence of chick embryo cells, sugar was a necessary constituent of the fluid medium. In the first instance, the glucose was omitted from the Tyrode's solution, serum still forming a constituent of this medium. In the first two passages there was a considerable fall in the virus concentration, but on the third passage and thereafter the virus grew in the serum Tyrode's solution as readily without as with glucose. Growth of the virus also occurred when the glucose in the Tyrode's solution was replaced by similar amounts of lactose or maltose. Since, however, a small amount of glucose was almost certainly present in the normal serum, it seemed possible that this glucose might provide the quantity necessary for growth of the virus. Finally, therefore, the virus was grown in the absence of serum in a Tyrode's solution containing no carbohydrate of any sort. Twenty passages were successfully carried out in this medium. The energy requirements of the chick embryo cells could, therefore, only be met by substances stored in the cells. How far these stored constituents suffice for the active metabolism of the cells over a period of four days is a question on which further observations are required.

VARIATION IN THE FLUID AND CELL CONSTITUENTS OF THE TISSUE CULTURE MEDIUM.

In this series of experiments attempts were made to replace the embryonic cells by substances which might conceivably be formed by the metabolism or breadkown of the tissue cells.

Chick embryo cells were suspended in Tyrode's solution and to the suspension was added liquor trypsini, in a proportion of 1 in 20. The mixture was then placed in the incubator at 37° C. for 12 hours. At the end of this time the trypsinized cell suspension was treated in a number of ways. To one set of flasks was added 1 c.c. of trypsinized fluid together with the cellular debris still remaining in the mixture. A second set of flasks received the trypsinized supernatant fluid after centrifuging at 3000 r.p.m. for 5 minutes to remove coarse debris. A third set of flasks received the same trypsinized fluid heated to 56° C. for 30 minutes to inactivate any trypsin still present. In order to show that these fluids were not themselves toxic to the yellow fever virus, they were all added to flasks containing the standard medium, which had already been incubated for 3 days and were subsequently incubated for 24 hours. The virus failed to exhibit growth in any of the three sets of tubes containing no embryo cells, but was present in normal concentration in the control tubes. Under the experimental conditions, therefore, no indication

was obtained that tryptic digestion of chick embryo cells liberated any substance or substances which could replace the embryo cells as growth factors.

Since claims have been made that essential growth factors can be liberated from tissue cells by exposure to ultraviolet light, chick embryo cells spread out in a Petri dish and just moistened with Tyrode's solution were exposed for 30 minutes to a mercury vapour lamp at a distance of 18 in. The tissues were then ground up with sterile sand and Tyrode solution added to form a suspension of cells approximately 1 in 10. The suspension was lightly centrifuged at 3000 r.p.m. for 5 minutes, and 1 c.c. of the supernatant fluid was added to the serum Tyrode mixture in place of the intact cells. No growth and no survival of virus occurred in the absence of embryonic cells, but growth occurred in flasks containing the extract together with embryonic cells, thus showing that the extract was not itself toxic to the yellow fever virus.

Finally the effect of replacing the embryonic cells by a complete amino-acid mixture was tested. The amino-acid mixture was made up as follows:

l-tryptophane . . 0·1 g.	*dl*-alanine . . 0·1 g.	*dl*-lysine di HCl 0·1 g.
l-cystine . . 0·2 g.	*dl*-leucine . . 0·5 g.	*l*-histidine HCl 0·2 g.
dl-phenylalanine . . 0·1 g.	*dl*-*iso*leucine . . 0·1 g.	*d*-arginine . 0·1 g.
glycine . . 0·5 g.	*dl*-*nor*leucine . 0·1 g.	*dl*-proline . 0·1 g.
dl-methionine . . 0·1 g.	*dl*-aspartic acid . 2·5 g.	*l*-oxyproline . 0·1 g.
dl-valine . . 0·5 g.	*dl*-serine . . 0·1 g.	*dl*-threonine . 0·1 g.
dl-glutamic acid . . 2·5 g.	*dl*-tyrosine . . 0·1 g.	

Distilled water to 1 litre.

When this mixture was added in 1 c.c. amounts to the standard medium containing embryo cells, growth of the virus occurred quite as readily as in the absence of the amino-acids, thus showing that they were not toxic to the virus. In the absence of embryo cells, however, the amino-acid mixture added to the serum Tyrode's solution failed to support the growth of the virus.

It thus appears that the yellow fever virus does not synthesize itself when provided with a complete amino-acid mixture. A number of bacteria, however, are known to require specific growth factors which are not necessarily amino-acids. Up to the present, the action of only two such specific growth factors has been tested on the growth of the yellow fever virus. Thiamine and nicotinic acid added either alone or together to the amino-acid mixture in amounts of 10γ per 10 c.c. of the mixture, and then combined with the serum Tyrode mixture, failed in the absence of embryonic cells to allow either survival or growth of the yellow fever virus. In the presence of embryonic cells growth of virus occurred showing that the factors were not in themselves toxic to the virus. Further observations are being carried out in the hope that eventually by the addition of one or more growth factors to the amino-acid mixture a synthetic medium may be obtained which will support growth of the yellow fever virus in the absence of embryonic cells.

DISCUSSION.

Although it has proved impossible up to the present to find a medium which supports the growth of the yellow fever virus in vitro in the absence of cells, certain results have been obtained which throw light on the role of cells in the growth of the yellow fever virus, since neither embryonic cells killed

by heat nor inert substances on which the virus can be adsorbed are capable of replacing living cells. It is thus probable that the function of the living cells is not merely to provide a physical basis for the multiplication of the virus. Since, however, the virus can multiply in the presence of cells suspended in a simple saline solution which provides very little, if anything, for the energy requirements of the cells, the virus must be able to multiply in the presence of cells whose metabolism is either nil or at least is proceeding very slowly.

The cells, even if their metabolism is at a low ebb, cannot, however, be replaced by a complete amino-acid mixture. Nevertheless, as in the case of many bacteria, the addition of an essential growth factor or factors to this basal amino-acid mixture may eventually provide a medium which will support the growth of the virus in the absence of living cells. On the other hand, the material of which the virus is composed may be derived from cell substances which are not necessarily broken down as far as the amino-acid stage. Up to the present, however, there is little knowledge of the enzymatic constitution of viruses, although Macfarlane and Salaman (1938) have found that vaccinia virus contains catalase and a small amount of phosphatase. Possibly the obligatory intracellular existence of viruses is due to the fact that possessing few or no enzymes of their own, they are forced to borrow the enzyme systems of the cells they parasitize in order to build up their own specific substance.

SUMMARY.

Prolonged growth in vitro of the attenuated tissue culture yellow fever virus 17D took place in a serum Tyrode medium containing a high concentration of chick or mouse embryonic nervous tissue without detectable increase in the neurotropism of the virus. No growth was obtained in adult mouse brain tissue.

In addition to mouse or chick embryo tissues, the virus grew readily in vitro in serum Tyrode with human placental tissues, and with mouse carcinoma or sarcoma tissues.

No growth of the tissue culture virus or of a neurotropic strain was obtained when embryonic cells were replaced by sterile larval tissues of *Aëdes aegypti*.

Chick embryo cells heated for half-an-hour at 56° C. failed to support growth of the virus.

The virus failed to grow in the serum Tyrode medium in the absence of animal cells but with the addition of the following: (*a*) An extract of trypsinized embryonic cells, (*b*) an extract of embryonic cells exposed to ultra-violet light, (*c*) a complete amino-acid mixture, (*d*) a complete amino-acid mixture with the addition of either or both thiamine and nicotinic acid, (*e*) living yeast cells or living *B. coli*, (*f*) minced Seitz EK filter pads.

The virus grew in a Tyrode medium with embryonic cells in the absence of serum, and in a Tyrode serum medium with embryonic cells when either the Tyrode solution contained no glucose or the glucose was replaced by either maltose or lactose.

The virus also grew readily with embryonic cells in a Tyrode medium containing no glucose and without the addition of serum, that is to say in a

fluid medium containing inorganic salts, but no organic substances capable of providing for the energy requirements of the cells.

Our thanks are due to Mr. A. E. Green for carrying out numerous routine cultures, a labour extending over many years.

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IMMUNIZATION EXPERIMENTS WITH ARTIFICIAL COMPLEXES FORMED FROM SUBSTANCES ISOLATED FROM THE ANTIGEN OF *BACT. SHIGAE*.

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THE results of recent work (Morgan and Partridge, 1939*b*, 1940) have shown that the dominant antigenic complex associated with the " smooth " form of *Bact. shigae* consists of three major components—a polysaccharide, a phospholipin and a polypeptide. The presence of other minor components, however, was not excluded.

The antigenic material was extracted from the bacterial cell by means of cold, anhydrous diethyleneglycol (Morgan, 1937), and there seemed good reason for believing that the antigenic complex which was ultimately isolated consisted essentially of a homogeneous molecular complex that possessed the immunological properties of the " smooth " somatic antigen of the intact bacterial cell. By the dissociating action of cold neutral formamide it was possible to split the antigenic molecular complex into its components. Thus, the phospholipin was readily removed and a complex of the specific polysaccharide and the polypeptide, which showed powerful antigenic properties, was obtained (Morgan and Partridge, 1939*a*). Repeated formamide treatment of this material resulted in a more extensive breaking down of the complex into polysaccharide and polypeptide components. A number of preliminary experiments were carried out in order to determine whether the three isolated components were antigenic when tested by themselves and whether it was

possible to re-constitute the components so as to form a complex which would possess antigenic properties similar to those of the original antigen.

In the present communication, details of the immunological aspects of the work are given. Owing to the difficulty of producing in laboratory animals an experimental infection with *Bact. shigae* it has not been possible to show that the artificial antigenic complex described in this paper will induce the formation of protective antibody. Since most of the experiments described in this paper were completed, however, a method for the titration of the antibacterial immune-body in an immune serum against *Bact. shigae* has been elaborated by Henderson and Steabben (unpublished work). A number of our sera have recently been examined by this method and the results will be given in a paper by these authors. In this communication it has only been possible to express any immune response observed in terms of *in vitro* reactions—specific precipitation, agglutination and, in part, complement fixation with a " Shiga " suspension or with the primary diethyleneglycol extraction product. Some observations on the production of " Shiga " heterophile immune-body are also included.

METHODS.

Preparation of Antisera.

Mature rabbits were inoculated intravenously at intervals of three days with doses of antigenic material varying from 0·01 mg. to 0·05 mg. A course of immunization consisted of three doses and a sample of blood was taken six days after the last dose. A second, similar course of injections was usually given.

Specific Precipitation Reaction.

The undiluted immune serum (0·2 ml.) was added to dilutions of " Shiga " specific polysaccharide in 1 per cent. saline solution (0·2 ml.) or to dilutions of " Shiga " polypeptide (at pH 7·5 in buffer phosphate-saline solution) in small test tubes, and the mixture, after shaking, incubated at 37° for two hours. The test was read after the tubes had stood at 0° C. for 18 hours.

Agglutination Test.

One drop of a heavy suspension of formolized " smooth " *Bact. shigae* was added to dilutions of the antisera (1 ml.) and the test heated at 45° C. for 4 hours. The test was read after standing 18 hours at room temperature. In the tables, complete agglutination is denoted by the figure 4, 1 representing a degree of agglutination just noticeable with the aid of a hand lens. Positive controls were included in each test and the normal sera served as negative controls.

Haemolysin Test for " Shiga " Heterophile Antibody.

The serum dilutions (1 ml.) were placed in small test-tubes and complement added (0·5 ml. of a 1 : 10 dilution of tested guinea-pig serum in saline). A 3 per cent. suspension of washed sheep red cells was now added (0·5 ml.) and the mixture was heated for 1 hour at 37° C. in a water-bath. The test was read after 18 hours at 0° C. A suspension of red cells (0·5 ml.) in saline (1·5 ml.) and a suspension of red cells in complement (0·5 ml., 1 : 10 dilution) and saline (1 ml.) were put up as controls, and all the appropriate normal sera were tested at the same time in each haemolysis experiment performed.

Complement Fixation Test.

The sera were examined according to the method described by Henderson (1939). At least 2 M.H.D. of complement were used in each fixation experiment and the concentration of antigen used in the test was not greater than one-fourth of the smallest amount which inhibited lysis. Readings were taken after one hour at 37° C. and again after standing overnight at 1–2° C.

IMMUNIZATION EXPERIMENTS.

(1) *Immunization with the Polysaccharide-polypeptide Complex.*

Details of the method for the preparation of the primary diethyleneglycol extraction product have already been given (Morgan, 1937) and it was shown subsequently that this material, which gives rise to a heavily opalescent solution in water, dissolves in cold anhydrous formamide to give an almost clear colourless solution. Fractional precipitation of the formamide solution with ice-cold alcohol yields, in part, a less readily soluble fraction which is entirely free from phospholipin (Morgan and Partridge, 1939*a*). The resulting polysaccharide-polypeptide complex, which contains 18–22 per cent. of the latter component, is strongly antigenic. Intravenous doses of the material each amounting to only 0·02 mg., when given to rabbits (Table I, Animals 11, 12, 25 and 26), yield antisera which appear to be qualitatively and quantitatively similar to those prepared against the primary extraction product (Rabbits 43, 44, 515 and 517, Table I) or against vaccines prepared from the whole bacteria. The immune sera 11 and 12 gave slight but definite precipitation when tested with the polypeptide component of the antigen (see Table III). This result at once suggests that the phospholipin component is not essential for the manifestation of antigenicity in spite of the fact that it appears to be an integral part of the antigenic complex. In view of this conclusion an attempt was made (Morgan and Partridge, 1940) to remove the polypeptide component from the primary extraction product by means of trypsin digestion in order to study the immunological behaviour of a possible polysaccharide-phospholipin complex that might remain.

(2) *Immunization with the Polysaccharide-phospholipin Complex.*

Although tryptic digestion of the polysaccharide-polypeptide complex proceeds rapidly and results in an amino-acid-free polysaccharide preparation, digestion of the primary extraction product proceeds very slowly and cannot be induced to go to completion. The method, however, was successful in yielding a complex containing only 3–4 per cent. polypeptide, in which the phospholipin component (14–17 per cent. in different preparations) was still firmly bound. Details of this preparation have already been given (Morgan and Partridge, 1940). In some physical characteristics the complex was similar to the primary extraction product, yielding heavily opalescent suspensions in water which showed some signs of sedimentation after standing several weeks at 0° C. No phospholipin could be removed by extracting this material with ether or chloroform—solvents which dissolve readily the phospholipin component. As will be seen from Table II the product was only weakly antigenic (Rabbits Nos. 61 and 62), and it appeared probable that the material owed its slight activity to the 3 or 4 per cent. of contaminating polypeptide. A polysaccharide-polypeptide complex that was isolated in a different manner and which contained only 4–5 per cent. of the latter component was also found to possess slight but definite antigenic properties. In order to obtain further evidence in support of this conclusion, however, removal of the phospholipin from the complex was effected by dissolution of the material

TABLE I.—*Showing the Production of "Shiga" Agglutinins and Precipitins in Rabbits Inoculated with Substances Isolated from the "Shiga" Antigen.*

Nature of material injected.	Dose (mg.).	Number of doses.	Rabbit No.	Dilution of immune serum giving— precipitation with "Shiga" polysaccharide. 1:10^4.	1:10^5.	1:10^6.	agglutination with formolized suspension of *Bact. shigae.* 1:20.	1:40.	1:80.	1:160.	1:320.	1:640.	1:1280.
Primary extraction product	0·05	3	43	4	4	1	4	4	4	3	2	1	0
			44	4	4	1	4	4	4	4	3	3	1
			515	4	4	1	4	4	4	4	2	0	0
			517	2	3	1	4	4	4	3	1	0	0
Polysaccharide-polypeptide complex	0·02	3	11	2	3	2	4	4	4	4	4	4	3
			12	2	3	1	4	4	4	4	4	3	2
	0·02	6	25	4	4	1	4	4	4	4	4	4	2
			26	4	4	1	4	4	4	4	4	4	4
High viscosity polysaccharide component	0·05	6	69	0	0	0	0	0	0	0	0	0	0
			70	0	0	0	0	0	0	0	0	0	0
			92	0	0	0	0	0	0	0	..	..	..
			93	0	0	0	0	0	0	0	..	..	..
Polypeptide component	0·05	3*	84	0	0	0	0	0	0	0	0	0	0
	0·10	3†	85	0	0	0	0	0	0	0	0	0	0
	0·05	6	96	0	0	0	1	0	0	0	0	..	..
			97	0	0	0	1	0	0	0	0	..	..

* 1st course of inoculations.
† 2nd course of inoculations.

TABLE II.—*Demonstrating the Weak Antigenic Response in Rabbits Inoculated with a Phospholipin-polysaccharide Complex and with the Polysaccharide Material after Removal of Phospholipin.*

Nature of material injected.	Doses.	Rabbit No.	Dilution of immune serum giving— precipitation with "Shiga" polysaccharide. 1:10^4.	1:10^5.	1:10^6.	agglutination with formolized suspension of *Bact. shigae.* 1:20.	1:40.	1:80.	1:160.	1:320.	1:640.	1:1280.
(A) Phospholipin-polysaccharide complex*	1st course	61	0	0	0	1	1	0	0	0	0	0
	3 × ·02 mg.	62	0	0	0	1	0	0	0	0	0	0
	2nd course	61	2	3	2	4	3	2	1	0	0	0
	3 × ·05 mg.	62	0	0	0	2	0	0	0	0	0	0
(B) Polysaccharide prepared from (A) by formamide fractionation†	1st course	82	0	0	0	0	0	0	0	0	0	0
	3 × ·02 mg.	83	0	0	0	1	0	0	0	0	0	0
	2nd course	82	0	0	0	2	0	0	0	0	0	0
	3 × ·05 mg.	83	2	2	1	4	4	2	1	0	0	0

* Analysis: Phospholipin 14 per cent., polypeptide 3 per cent.
† Analysis: Phospholipin 0 per cent., polypeptide 4 per cent.

(500 mg.) in formamide (50 ml.), and precipitation of the polysaccharide component by addition of three volumes of ice-cold alcohol. The precipitate was again dissolved in formamide and the precipitation was repeated. The polysaccharide substance that was recovered dissolved in water to give an almost clear solution which showed little more than a faint bluish opalescence. Heating a portion of the material at 95° C. for three hours with 1 per cent. acetic acid yielded 4 per cent. of the insoluble polypeptide, but no phospholipin was found. The phospholipin-free material, however, when tested in rabbits (Table I, Animals 82, 83) gave results very similar to those obtained with the original complex which contained 14 per cent. phospholipin. It appears, therefore, that, although combination of the specific polysaccharide with phospholipin yields a complex which has the property of forming very large micelles in aqueous media and which displays a heavy opalescence which might be considered characteristic of aqueous solutions of bacterial antigens prepared by the diethyleneglycol method, the combination does not confer the property of antigenicity.

(3) *Immunization with an Undegraded "Shiga" Polysaccharide.*

The earlier methods of preparing the specific polysaccharide in a state of purity all involved the use of high temperatures or rather drastic chemical treatment (Morgan, 1931, 1936), which ultimately involved the use of 96 per cent. acetic acid and a method subsequently used (Morgan, 1937) consisted in heating at 100° the primary extraction product in 1 per cent. acetic acid for an hour or more, when the polypeptide and phospholipin components separated from solution as a flocculent precipitate and the clear aqueous supernatant contained the polysaccharide. This polysaccharide product, by suitable fractionation, could readily be obtained in a state of considerable purity. It dissolved readily in water to yield a clear mobile solution. In this form a 2 per cent. solution in $M/6$ citrate phosphate buffer at pH 7 containing sodium chloride (4 per cent.) had a relative viscosity of 1·96 at 34° C. (water = 1) (Ostwald viscometer).

The polysaccharide obtained by extensive formamide dissociation of the polysaccharide-polypeptide complex, however, while it was free from amino acids and its nitrogen content and optical rotation (1·9 per cent. $[\alpha]_{5461} + 100 \pm 5°$) were not significantly different from the low viscosity polysaccharide (N, 1·8 per cent. $[\alpha]_{5461} + 111°$), it dissolved much more slowly in water and gave rise to very viscous solutions (Morgan and Partridge, 1940). A 2 per cent. solution of the material in the same buffer-salt solutions as above had a viscosity of 42 (relative to water = 1) at 34°. As will be shown later, there is good reason for believing that much of the polysaccharide in this product is in an undegraded state. Like the disaggregated polysaccharide, however, this material forms specific precipitates with antibacterial "Shiga" immune serum and is not antigenic when tested in rabbits (Table I, Animals 69, 70, 92 and 93).

As the process of exhaustive fractionation of the polysaccharide-polypeptide complex with alcohol from formamide solution is somewhat laborious

and the ultimate yield of polysaccharide is low, a more economical method has been adopted for the production of the high viscosity polysaccharide.

Polysaccharide-polypeptide complex (400 mg. in 40 ml. water) was digested in the presence of chloroform with 4 ml. $M/5$ disodium hydrogen phosphate solution at 37° for 24 hours, successive small additions of commercial trypsin being made during this time. The mixture was then dialysed against distilled water at 0° until free from phosphate and shaken vigorously with a small quantity of chloroform. The emulsion was centrifuged and the aqueous supernatant layer, which now showed only a faint bluish opalescence, was decanted, concentrated to about 30 ml. and the polysaccharide precipitated by addition of alcohol. After washing thoroughly with absolute alcohol the precipitate was dissolved in 30 ml. of anhydrous formamide and once more fractionated by additions of ice-cold alcohol. The precipitate, after triturating with several portions of alcohol, was dissolved in distilled water and thoroughly dialysed at 0°, after which it was recovered by concentration *in vacuo* at 15° and finally evaporated to dryness *in vacuo* from the frozen state. The resulting polysaccharide was found to be similar in viscosity, nitrogen content and immunological behaviour to the samples of high viscosity polysaccharide isolated by simple formamide dissociation as described above.

(4) *Immunization with the Polypeptide Component.*

The method of degrading the primary extraction product or the polysaccharide-polypeptide complex by heating with 1 per cent. acetic acid still remains the only practicable means of obtaining the "Shiga" polypeptide component. As the method involves heating the material to 95° for several

TABLE III.—*Showing the Precipitation of "Shiga" Polypeptide with Sera Prepared against Different Antigenic Fractions.*

Antigen used in preparation of the rabbit immune sera.	Dose.	Rabbit No.	Precipitation tests. Dilution of "Shiga" polypeptide in saline. 1:10^3.	1:10^4.	1:10^5.
Polypeptide component	3 × ·05 mg.*	84	1	2	1
	3 × 1 mg.†	85	0	0	0
	6 × ·05 mg.	96	3	2	0
		97	2	2	0
Natural polysaccharide-polypeptide complex	3 × ·01 mg.	11	2	0	0
		12	2	0	0
Primary extraction product	3 × ·05 mg.	515	1	0	0
		517	1	0	0
Re-synthesized antigen (artificial polysaccharide-polypeptide complex)	6 × ·05 mg.	98	1	0	0
		99	3	3	1
		100	3	3	0

* 1st course of immunization.
† 2nd course of immunization.

hours, however, the possibility that the substance has been modified by this process must be recognized. Prepared in this manner, the polypeptide is soluble in dilute aqueous buffer solutions at pH 8·0 and above but is insoluble at an acid or neutral pH. In alkaline solution the material very slowly passes through a cellophane membrane and it must be concluded that its molecular size has a value approximately equal to the limiting size determined by the porosity of cellophane. It contains about 5 per cent. arginine, 8 per cent. tyrosine, 6 per cent. tryptophane and at least 12 per cent. glutamic acid. Sulphur appears to be absent. After acid hydrolysis the products gave about 3 per cent. reduction, determined as glucose by the method of Shaffer and Hartmann (1921), and a slightly higher figure was given when the orcinol method of Sørensen and Haugaard (1933) was employed. Further details of the chemical nature of this interesting component will be given in a later paper. Rabbits were immunized with the polypeptide material. The substance was dissolved in buffer phosphate at pH 8·0 and the appropriate dilutions were made in saline just before use. Most of the immune sera tested gave distinct precipitation with the homologous antigen (Table III, Animals 84, 85, 96 and 97). After an immunizing course of six doses of 0·05 mg. of the polypeptide, a trace only of " Shiga " agglutinins at 1 : 20 dilution was detected in two sera examined. Anti-polypeptide sera frequently gave definite although slight precipitation when tested with the primary extraction product and with the artificial polysaccharide-polypeptide complex. Two anti-polypeptide sera (Nos. 96 and 97) were pooled and the mixed sample was exhausted of homologous precipitins by absorption with polypeptide. The resulting serum failed to show any precipitins against the natural or artificial polysaccharide-polypeptide complex.

Guinea-pigs passively immunized (see Morgan, 1932) with 5 ml. of a pooled immune-serum prepared against the polypeptide showed definite signs of anaphylaxis when 1 and 2 mg. of polypeptide were given intravenously. A dose of 5 mg. caused very severe shock, but all the animals recovered.

(5) *Immunization with the Artificial Polysaccharide-polypeptide Complex.*

In view of the fact that the polysaccharide-polypeptide complex behaves in formamide solution as if it is in equilibrium with its components and further that the antigenic material retains its activity after standing in formamide solution for long periods, it was decided to investigate the possibility of reversing the dissociation process in that solvent. It was found that the polypeptide, when dispersed in a concentrated solution of the high viscosity polysaccharide in formamide, yielded on dialysis highly opalescent stable suspensions characteristic of the natural antigenic complex and that these suspensions were in fact potent antigens. The best reaction conditions so far determined are as follows :

The high viscosity polysaccharide (60 mg. N, 1·9 per cent.), prepared in this instance by a combination of tryptic digestion and formamide dissociation, was dissolved in 1·5 ml. formamide (m.p. + 2°), and polypeptide (40 mg. N, 12·2 per cent.) was added as a dry powder. The powder rapidly swelled to a

clear gel and part appeared to pass into solution. A further portion of formamide (1·5 ml.) was added and the dispersion was completed in a glass tissue grinder, which yielded an apparently homogeneous faintly opalescent solution. This was allowed to stand overnight at room temperature in order to attain equilibrium, and the volume was then increased to 5 ml. and the solution was allowed to stand for 6 hours at — 10°. The complex was precipitated from solution by addition of 15 ml. ice-cold alcohol and was triturated repeatedly with fresh additions of the same solvent. The precipitate was then dissolved in 10 ml. 1 per cent. saline and gave rise to a heavily opalescent solution. This was centrifuged for five minutes at 3500 r.p.m. in an angle centrifuge and the small precipitate, which appeared to be uncombined polypeptide, was rejected. The complex was then precipitated from the aqueous solution by addition of 20 ml. alcohol. The precipitate redissolved in water to yield a suspension of unimpaired stability and was dialysed for eight days at 0° against frequent changes of distilled water. The results of immunization experiments with this artificial complex are given in Table IV (Rabbits 98, 99 and 100), and show that the artificial complex was strongly antigenic and that its properties as an antigen were similar to those of the primary extraction product or the whole bacteria. The sera gave distinct precipitation when tested against the polypeptide component (Table III, Animals 98, 99 and 100). The samples of polysaccharide and polypeptide used in this preparation were tested in other rabbits (Table I, Nos. 69, 70, 84, 85, 92 and 93) for control purposes, but neither preparation gave rise to any significant immune response as measured by specific " Shiga " agglutination, precipitation, complement fixation or haemolysin formation.

Various attempts had previously been made to effect a resynthesis of the antigen in aqueous solution (see below) using the highly purified, low viscosity polysaccharide as a starting material, but these had proved unsuccessful. In view of the success of the formamide method of resynthesis as described above, it was decided to repeat the process using the low viscosity polysaccharide. A sample of the latter material (N, 1·8 per cent. ; P, 0·18 per cent.) (60 mg.) was prepared from the native antigenic complex by 1 per cent. acetic acid hydrolysis at 100° and, after careful purification, was triturated with a specimen of the polypeptide component (20 mg.) in formamide solution. After precipitation with alcohol and dialysis to remove the remaining traces of formamide a very unstable suspension was obtained, the material being readily deposited when centrifuged at 3500 r.p.m. After drying in the usual manner, the resuspended material when tested in animals (Table IV, Nos. 86 and 87) did not prove to be antigenic. Another preparation gave a similar result.

Przylecki and his collaborators (Przylecki and Majmin, 1935 ; Przylecki and Refalowska, 1935) showed that certain amino-acid and protein-polysaccharide complexes may be prepared by mixing aqueous solutions of the components and precipitating the complex formed by subsequent adjustment of the pH of the mixture. Experiments were therefore begun with the object of ascertaining if the combination of " Shiga " polysaccharide with polypeptide could be secured by such methods and to test the antigenicity of the products formed. These experiments are still in progress, but the result of one attempt is now given.

TABLE IV.—*Precipitation and Agglutination Reactions of Rabbit Immune Sera Prepared against Different Artificial Complexes.*

Material used as antigen.	Animal number.	Number of doses and amount.	Precipitation test. Dilutions of polysaccharide. 1:10^4.	1:10^5.	1:10^6.	Agglutination test.* Dilutions of immune serum. 1:20.	1:40.	1:80.	1:160.	1:320.	1:640.	1:1280.
Complex prepared in formamide from high viscosity polysaccharide and "Shiga" polypeptide	98	3 × ·05 mg.	4	4	2	4	4	4	4	3	2	1
	99		4	4	2	4	4	4	4	3	2	1
	100		3	4	1	4	4	4	3	2	1	0
Complex prepared from high viscosity polysaccharide and "Shiga" polypeptide in aqueous solution	94	3 × ·05 mg.	3	4	2	4	4	3	0	0	0	0
	95		3	4	2	4	4	4	3	2	1	0
Complex prepared from low viscosity polysaccharide and "Shiga" polypeptide by trituration in formamide	86	3 × ·05 mg. + 3 × ·01 mg.	0	0	0	0	0	0	0	..	..	..
	87		0	0	0	0	0	0	0	..	..	..
High viscosity "Shiga" polysaccharide adsorbed on collodion particles	101	3 × ·05 mg.†	0	0	0	0	0	0	0	..	..	..
	102		0	0	0	0	0	0	0	..	..	..
High viscosity "Shiga" polysaccharide adsorbed upon denatured rabbit serum proteins	103	3 × ·05 mg.†	0	0	0	0	0	0	0	..	..	..
	104		0	0	0	1	0	0	0	..	..	..
	105		0	0	0	0	0	0	0	..	..	..

* Formolized whole culture of "smooth" *Bact. shigae.*
† Calculated as specific polysaccharide.

The polypeptide (20 mg.) was dissolved in $M/5$ disodium hydrogen phosphate buffer solution (0·5 ml.) and a 2 per cent. aqueous solution of the high viscosity polysaccharide (2 ml.) was added. The mixture after standing for a short time was then dialysed in a cellophane bag against acetate buffer at pH 6. A flocculent precipitate resembling unchanged polypeptide came out of solution after a few hours' dialysis. The mixture was then dialysed against distilled water and the precipitate and supernatant fluid, after solution in saline at pH 8·5, were tested in rabbits. The material proved to be antigenic (Table IV, Animals 94 and 95), but there is little doubt that the association process is inefficient under the conditions described and that the mixture contained only a relatively small proportion of combined material.

(6) *Immunization with Undegraded Polysaccharide Adsorbed on Non-specific Substances.*

Many experiments have appeared in the literature (Landsteiner, 1936) in which bacterial and other polysaccharides have been adsorbed upon inert material and claims have been made that in some cases an antigenic product has resulted (Zozaya, 1931, 1932; Zozaya and Clark, 1933; Prášek and Prica, 1933; Jacobs, 1934). Experiments of this kind are still being undertaken, but the results so far obtained using two non-specific carriers only have been entirely negative. Animals 101 and 102 shown in Table IV were inoculated with a product obtained by adsorbing the high viscosity "Shiga" polysaccharide upon collodion particles according to a technique very similar to that described by Zozaya (1932). The particles were made by pouring a 5 per cent. solution of collodion (Schering-Kahlbaum) in aqueous acetone into a large volume of distilled water which was violently agitated during the addition. The larger particles were allowed to settle under gravity and the heavy suspension of stable particles was decanted and dialysed free from acetone. Sufficient of the collodion suspension was added to a dilute solution of the high viscosity polysaccharide to remove by adsorption practically the whole of the polysaccharide originally present. The extent of the adsorption was determined by precipitin tests performed upon the filtrate after removing the collodion particles by centrifugation in the presence of a low concentration sodium chloride.

Heat denatured normal rabbit serum protein was used as the carrier substance in the second experiment. The protein employed was obtained by heating dilute serum, buffered at pH 4·6, for 20 minutes at 100°. The denatured protein was removed by centrifugation, dissolved in dilute alkali and reprecipitated by cautious addition of dilute acetic acid. This process was repeated twice and 3·5 mg. of the substance, dissolved in dilute alkali (1 ml.), was added to a 1 per cent. solution of the high viscosity polysaccharide (1 ml.). The mixture was brought to pH 5 and the floccules dispersed by use of a glass tissue grinder. The heavily opalescent suspension was adjusted to pH 8 and allowed to stand at 0° overnight; only part of the suspension remained stable. The material given as a whole in 1 per cent. saline did not prove to be antigenic (Table IV, Animals 103, 104 and 105).

COMPLEMENT FIXATION TESTS.

Complement fixation tests have been carried out with anti-sera prepared against the natural and artificial antigenic complexes and two of the components of the whole antigenic complex. Using a dried culture of *Bact. shigae* as antigen and the sera of rabbits immunized with (1) the polypeptide component and (2) the high viscosity polysaccharide, no appreciable fixation of complement could be demonstrated. On the other hand, strong complement fixation, using 2 M.H.D. of complement, occurred when " Shiga " organisms were incubated with immune sera prepared against artificial polysaccharide-polypeptide complex and, as was to be expected, against the sera of rabbits immunized with a formolized whole culture of *Bact. shigae*. Complement fixation experiments were also made in which the artificial polysaccharide-polypeptide complex was employed as antigen in place of the " Shiga " organisms. Some fixation occurred with the homologous immune sera but the binding of complement was much less intense than was anticipated. Similarly, sera prepared against a whole culture of *Bact. shigae* only fixed complement moderately in the presence of the artificial polysaccharide-polypeptide complex. The weakness in the complement binding capacity of the artificial complex may be connected in some manner with the state of dispersion of the antigenic material. Control tests which employed (1) a suspension of *Bact. typhosum* and (2) an aqueous extract of " rough " *Bact. shigae* with the above anti-sera showed no fixation of complement. Similar negative results were obtained with anti-polysaccharide and anti-polypeptide sera and the two non-specific antigen controls. In a further series of tests an aqueous suspension of " rough " " Shiga " organisms was employed as antigen and was found to give marked fixation of complement in the presence of anti-sera induced by a formolized whole culture of the " smooth " variant of *Bact. shigae*. Furthermore, anti-sera elicited by the artificial polysaccharide-polypeptide complex likewise showed definite fixation of complement when the " rough " organism was used as antigen. These results are difficult to explain and further work on this aspect of the problem is being undertaken. It should be recorded, however, that the " rough " antigen employed was in the form of a very unstable suspension and sedimentation occurred during the performance of the test.

THE PRODUCTION OF " SHIGA " HETEROPHILE ANTIBODY.

The examination of the sera of rabbits immunized with the polysaccharide and polypeptide component showed that only a trace of haemolytic antibody against sheep red cells was produced. The increase was irregular and in most instances where it occurred the end point of haemolysis was moved to the next higher dilution only ; such slight increases have not been accepted as evidence that the material under consideration was able to induce the formation of " Shiga " haemolytic antibody. The immune sera produced after immunization with a preparation of polysaccharide-phospholipin showed the presence of heterophile immune body, but subsequent analysis of the complex showed that it contained 2–3 per cent. of the polypeptide component. The production of

haemolytic antibody in the two rabbits immunized was small—a trace of haemolysis at 1 : 200—and most probably arose as a result of the contamination of the complex with native antigen. The artificial complex composed of low viscosity polysaccharide and polypeptide component which had failed to induce the formation of specific " Shiga " agglutinins likewise failed to stimulate the production of " Shiga " heterophile immune-body. On the other hand, the corresponding complex made from high viscosity polysaccharide induced a high titre (1 : 2000) of haemolytic antibody in 2 out of 5 of the rabbits immunized. The remaining three animals showed only a slight response. In view of the irregular production of heterophile antibody in the sera of rabbits immunized with *Bact. shigae* it may be concluded that the artificial polysaccharide-polypeptide induces the formation of haemolytic immune-body as readily as does the natural " Shiga " somatic antigen.

DISCUSSION.

Immunization experiments with degradation products of the dominant antigenic complex associated with the " smooth " form of *Bact. shigae* are described and the results are compared with those obtained following immunization with the intact bacterial cells. The results indicate that a partially degraded antigen, which consists of a polysaccharide-polypeptide complex, behaves similarly to the native antigen and engenders " Shiga " agglutinins and precipitins. An excellent antigenic response is given following the administration of three doses each of about 0·02 mg. (see also Morgan, 1937, table ii, p. 2000) of either of these preparations. No evidence was obtained that the phospholipin component of the original antigenic complex plays any significant part as a structure essential for the manifestation of antigenicity, or contributes to the determination of the serological specificity of the resulting immune-body. A partially degraded native antigen which contained about 14 per cent. of phospholipin and only 3 per cent. of polypeptide component combined with the specific polysaccharide proved to be a poor antigen and its limited antigenic activity appeared to be associated with the presence of the polypeptide component since subsequent treatment of the complex, which resulted in the elimination of the phospholipin, gave rise to a material that consisted of almost pure polysaccharide combined with the 3–4 per cent. of polypeptide. This complex possessed the weak antigenicity of the original phospholipin-rich substance and, as is shown in Table II, neither the complex possessing 14 per cent. of phospholipin nor the phospholipin-free material induced a significant titre of specific agglutinins or precipitins after the first course of immunization, that is, after three doses of 0·02 mg. of the preparations.

Immunization experiments have also been carried out with two components of the native antigen, the polysaccharide and the polypeptide. The former substance failed to induce the formation of demonstrable immune-body against the primary extraction product or against the polysaccharide. The antisera induced after immunization with polypeptide, however, when examined in precipitation tests against the polypeptide component itself showed definite activity. These sera also gave a definite although slight precipitation when

tested with the native and artificial polysaccharide-polypeptide complex. The primary extraction product and the partially dissociated native antigen composed of polysaccharide and polypeptide components also induced the formation of precipitins which reacted with the purified polypeptide, and the artificial antigen, composed of polysaccharide and polypeptide, likewise elicited the production of polypeptide precipitins. It has been observed, however, that immune sera prepared against the whole organism do not yield precipitates when mixed with a solution of the polypeptide and guinea-pigs passively immunized with these sera showed no signs of anaphylaxis when 2 mg. of polypeptide was given intravenously. A dose of 5 mg. given intravenously produced a very slight shock ; the animals appeared quite normal after 5 minutes. The primary extraction product (the polysaccharide-polypeptide-phospholipin complex) induces the formation of only weak precipitins against the polypeptide. On the other hand, the smaller complexes, the natural and artificial polysaccharide-polypeptide complexes, induce precipitins reactive against the polypeptide much more readily. It is clearly impossible, from the evidence given, to form any very definite ideas as to the specific immunological function of the polypeptide in the natural antigen. The results, however, indicate that as it exists in partially dissociated native antigen or in the artificial polysaccharide-polypeptide complex, the polypeptide plays a definite rôle in inducing the formation of specific polypeptide receptor sites on at least a part of the immune-bodies formed.

Immunization experiments with haptens, the components of antigens that possess serological activity but which exhibit no significant antigenicity, have been undertaken by many workers. For purpose of discussion here only bacterial haptens need be considered, although a much greater number of investigations of this type have been concerned with the activation of the hapten of the Forssman heterophile antigen. Zozaya (1931, 1932) and Zozaya and Clark (1933) reported extensive experiments in which bacterial polysaccharides were adsorbed on collodion particles, bacteria and dextran, a nitrogen-free polysaccharide from *Leuconostoc mesenterioides*. Zozaya concluded that the polysaccharide hapten of *Bact. shigae*, which itself was not antigenic, induced the formation of specific " Shiga " agglutinins in rabbits after it had been adsorbed on collodion particles. We have carried out several similar experiments with the " Shiga " polysaccharide preparations described in this paper, but in these experiments no demonstrable anti-hapten bodies were formed. Prásĕk and Prica (1933) and Jacobs (1934) have also reported failure to engender specific agglutinins in the sera of rabbits immunized with the non-antigenic polysaccharide haptens of the antigens of rhinoscleroma bacilli and *V. cholerae* which had been adsorbed on collodion particles and charcoal respectively. Spassky and Danenfeldt (1936) have claimed that the specific precipitate formed on the addition of the purified polysaccharide hapten of *Bact. typhosum* to an anti-typhoid serum, when given intravenously to rabbits, induced the formation of specific " O " precipitins and agglutinins. The sera of rabbits immunized with the insoluble floccules produced by the interaction of high viscocity " Shiga " polysaccharide with its homologous immune serum have been examined by us, but no immune body demonstrable by precipitation or agglutination tests was present.

For some time past Landsteiner (1936) has believed that the antigenicity demonstrated by Landsteiner and Simms (1923) following the injection of hapten-protein mixtures into rabbits is due to a loose combination being formed between the non-antigenic polysaccharide hapten and the antigenic protein. The experiments described in this paper would support this contention, for it is clear from the experimental evidence given that the high viscosity " Shiga " polysaccharide can be made to combine under almost physiological conditions (aqueous solution at room temperature) with a polypeptide-like body and yield a complex that shows strong antigenic properties. Furthermore, the antigenic complex so formed is readily dissociated into its non-antigenic components by the action of an anhydrous neutral solvent such as formamide.

It is conceivable that the property of certain polysaccharides to react with polypeptides (or proteins) and give rise to antigenic complexes, the specificity of which is largely determined by the carbohydrate component, is of wide occurrence. Sordelli and Mayer (1931) have reported that bacteria grown on agar render the agar antigenic and produce anti-agar immune-bodies in animals immunized with the organisms. This observation has been repeatedly confirmed by many other workers. Similarly, Uhlenhuth and Remy (1933, 1934) found that gum-arabic was rendered antigenic in the presence of certain protein-like substances that were present in the crude gum-arabic preparation. The purified gum-arabic was itself not antigenic. More recently Seideman (1940) has reported that animals immunized with gum-arabic preparations that yielded positive protein reactions give rise to immune bodies reactive against the non-antigenic and purified gum arabic. These examples show clearly that under certain conditions polysaccharides in association with proteins form antigenic complexes in much the same manner as has been described in this paper for the components of the antigen of *Bact. shigae*.

The experiments described in this paper are being extended to include the combination of " Shiga " polysaccharide with other naturally occurring proteins and with non-antigenic polypeptide complexes derived from commercial peptones. To determine the simplest and most gentle conditions under which non-antigenic polysaccharide can be converted into full antigens is considered to be of extreme importance, for the knowledge of antigen formation thus gained might help to explain the widespread occurrence in the plant and animal worlds of antigenic complexes, the immunological specificity of which are largely associated with polysaccharide.

SUMMARY.

1. The results of immunization experiments are given in which rabbits were inoculated with two components of the antigen of *Bact. shigae* and with an artificial complex built up from these components.

2. The specific polysaccharide component failed to induce any demonstrable immune-body. The polypeptide-like component engendered homologous precipitins of low titre but no significant agglutinins for *Bact. shigae*.

3. The artificial polysaccharide-polypeptide complex induced the formation

of potent immune-body which agglutinated *Bact. shigae* to high titre, precipitated with the specific polysaccharide and fixed complement in the presence of " Shiga " antibacterial sera.

4. The specific polysaccharide adsorbed on two non-specific substances was not rendered antigenic.

We have to thank our colleague, Dr. D. W. Henderson, for carrying out the complement fixation tests.

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ON THE RELATIONSHIP OF LESIONS OF THE SUPRARENAL GLAND TO DIPHTHERITIC TOXAEMIA.

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IT has been suggested by a number of writers that an important part of the effects of diphtheritic toxaemia is caused by damage by the toxin to the endocrine glands, particularly to the suprarenals. The following may be quoted in this connection: Rosenthal (1914); Elkeles and Hermann (1927 *a*, *b*); Mikami (1925); Lawrence and Buckley (1927); Bamberger, Neuer and Oelkers (1938); Thaddea (1935); Zwemer and Jungeblut (1935). In view of this evidence, there is a *prima facie* case for supposing that in diphtheritic toxaemia the brunt of the damage falls on the suprarenal glands. If this be so, the question is one of great practical importance in view of the increasing opportunities for replacement therapy. The question has been more fully discussed elsewhere (Holmes, 1939), and the conclusion reached that it was difficult to accept the view that endocrine damage accounted for the hitherto recognized metabolic disturbances in toxaemia; and the administration of large doses of suprarenal cortical hormone to rabbits previously treated with diphtheria toxin has been shown only partially to restore their power to deposit liver glycogen from injected lactate or to remove injected lactate from the blood stream (Dawson and Holmes, 1939). The development of the technique for investigating the metabolism of isolated tissues, provided it is used in conjunction with investigations on the whole animal and, where opportunity offers, with clinical observation, makes it easier to investigate the changes brought about by pathological agents. It has been found that diphtheritic toxaemia causes certain alterations in the metabolic events which take place in isolated slices of liver, as well as in those (e.g. glucose tolerance, response to insulin, power to remove lactic acid from the blood stream) which occur in the whole animal. It seemed, therefore, desirable for two reasons to obtain further evidence about the effect of the suprarenal upon metabolism: first, because further information was needed about the possible role of suprarenal deficiency in toxaemia; second, because it is not yet clear how far the behaviour of isolated slices of normal organs may depend upon the influence of hormones, for while no additional hormone can reach the cells during the progress of the experiment (as presumably it would if they were still supplied by the blood), yet they probably carry with them a certain supply of these substances.

The most marked metabolic features of diphtheritic toxaemia which we

have so far observed in liver slices (Cross and Holmes, 1937 ; Dawson and Holmes, 1939) have been, in rabbits, 48 hours after a dose of toxin usually lethal in 72 hours, (*a*) a marked diminution of the power of the liver to form carbohydrate from lactate, pyruvate and alanine ; (*b*) a marked diminution in its power to remove lactate and pyruvate ; (*c*) diminution in its power to oxidize lactate and pyruvate, coupled with a fall in the respiratory quotient. In less severely toxic animals (i.e. 24 hours after injection of toxin) the liver slices show only slight diminution in the power of gluconeogenesis. They will still form glycogen from added glucose, but their power to cause the disappearance of added lactate is markedly impaired ; so also is the rate of disappearance of injected lactate from the blood stream of the intact animal. Many authors have noted a decrease in glucose tolerance progressive during the course of the toxaemia.

If these results spring primarily from damage inflicted by the toxin on either the cortex or on the medulla of the suprarenal glands, it follows that similar results should be obtained in experiments with liver slices of animals from which the suprarenal glands have been removed.

The behaviour of liver slices taken from suprarenalectomized animals cannot be influenced by the effect of either the cortical or medullary hormones of the glands, though it obviously may be affected by other hormones (e.g. insulin, thyroxin, or the secretions of the anterior pituitary), and therefore such experiments should help to show how far the behaviour of isolated liver slices depends upon intrinsic properties of the cells, and how far it is influenced by the hormones which have been brought to it by the blood before the animal was killed.

In view of the results of Ostern, Herbert and Holmes (1939) on the phosphorylysis of glycogen in normal rabbit liver brei, it was thought desirable to investigate also phosphorylysis in the livers of suprarenalectomized rabbits. Verzár (Laszt and Verzár, 1937) and his school have maintained that phosphorylation of carbohydrates and fats depends upon the presence of the suprarenal cortical hormone. The phosphorylations which he studied, however, appear to have been definitely aerobic processes, while the phosphorylation of liver glycogen takes place both in the absence and in the presence of oxygen.

EXPERIMENTAL.

Rabbits of weight 1000–2000 g. were used. They were always starved for the 24 hours preceding the experiment. Suprarenalectomy was performed by a two-stage operation under ether, the incision starting at the level of the eighth costal cartilage and running parallel to the ribs, and for as far as was necessary down the abdominal wall. The right gland was always removed first, since it presents more difficulty than the left. Not less than a week, and usually considerably more, elapsed between the two operations. After the first operation the animals were given plentiful supplies of 5 per cent. glucose in 0·9 per cent. sodium chloride for a few days. They usually recovered well, and the wound healed without difficulty. The removal of the second suprarenal (which was generally seen to be much hypertrophied) usually left the animal in very poor condition. At the end of 24 hours, in spite of subcutaneous

and/or intravenous sodium chloride, the animals were mostly extremely weak, and hypoglycaemic. A number of them were therefore killed at this stage, and the metabolism of the liver slices examined. If, however, after the removal of the second suprarenal they were given " Eucortone " or other active cortical extract (one 1 c.c. dose intramuscularly at the end of the experiment and a further 0·5 c.c. 4–5 hours later), they recovered well, eating well (though generally losing weight), in spite of receiving no hormone after the first 24 hours after operation. They were used for experiment at various times, but not less than 4 days, after receiving their last dose of hormone. These animals were on the one hand free from the effects of shock and anaesthesia, and on the other were quite certainly lacking in all of the hormones normally supplied by the suprarenal, since careful post-mortem examinations were made, and any suspicious tissue sectioned. In one case only (one of the series taken 24 hours after the operation) was any suprarenal tissue found.

The chemical methods employed have been described in previous communications (Cross and Holmes, 1937 ; Dawson and Holmes, 1939). The only modification introduced was the employment of the cerium sulphate method of glucose determination for estimating sugar in the final stage of the measurement both for glycogen and for fermentable carbohydrate. It is both more sensitive and more convenient for this purpose than the Hagedorn and Jensen method. The Hagedorn and Jensen method was retained for blood sugar estimations.

Since it seems likely that the results obtained on animals used within 24 hours of the removal of the second suprarenal are complicated on the one hand by the effects of anaesthesia and operative shock, and on the other by the fact that it cannot be maintained with confidence that the tissues had not still sufficient hormone, they will not be discussed in great detail. Nevertheless, it may be pointed out that it was at this stage, when in poor condition, that the animals most closely resembled those which were suffering from severe toxaemia, in that the liver slices formed glycogen from added glucose, but formed little carbohydrate from added lactate.

In several instances glucose tolerance tests were performed both before and on the day following the removal of the second suprarenal. In no case was there any decrease in glucose tolerance, though there is ample evidence that decreased glucose tolerance very constantly accompanies all stages of diphtheritic toxaemia. Nor was there any decrease in the power of the liver brei to phosphorylate glycogen. Fig. 1 shows the result of glucose tolerance tests on two such animals. The dose of glucose was 0·5 g./kilo, injected intravenously as a 25 per cent. solution in water. The results are expressed as percentage change in blood sugar.

Slices of liver from these animals, shaken for one hour at 37° C. in Krebs bicarbonate medium containing 1 per cent. glucose, saturated with 95 per cent. O_2 and 5 per cent. CO_2, showed in all but two instances the power to form glycogen from added glucose at a rate little different from that observed with the tissue from normal animals.

In the case of animals which had been treated with cortical extract for the first 24 hours, and had survived the removal of the second suprarenal for 4 days or longer, the power of the slices to form glycogen was undoubtedly

TABLE I.—*Glycogen Formation from Glucose by Liver Slices.*

0·5 g. slices shaken for 1 hour at 38° C. in 3 c.c. Krebs medium containing 1 per cent. glucose. Bicarbonate buffer. 95 per cent. O_2/5 per cent. CO_2 gas phase.

Normal.			Suprarenalectomized.				Toxæmic.			
	Increase.			Days after removal of second suprarenal.	Increase.			Duration of toxæmia (hours).	Increase.	
Date.	mg./g. liver.	mg./kilo body weight.	Date.		mg./g. liver.	mg./kilo body weight.	Date.		mg./g. liver.	mg./kilo body weight.
16.i.40	2·5	83	12.x.39	7	0·58	16	18.iv.40	21	3·84	111
23.i.40	4·2	108	13.xii.39	31	0·87	27	15.iv.40	48	0·64	29*
8.iii.40	3·38	92	31.i.40	9	−0·93	−28	13.iv.40	48	2·50	81
20.iii.40	8·78	215	15.iii.40	8	0·18	5	11.iv.40	48	2·54	104
27.iii.40	6·6	203	6.iii.40	16	0·45	15	11.iv.40	48	0·05	1·6*
1.iv.40	11·28	326	16.ii.40	14	2·07	66	9.iv.40	48	5·8	161
10.iv.40	7·55	188	27.iv.40	4	1·36	35				
19.iv.40	1·96	46	18.v.40	5	−0·02	−0·5				
25.iv.40	6·6	172								
9.v.40	4·5	137								

* = animal in a collapsed state.

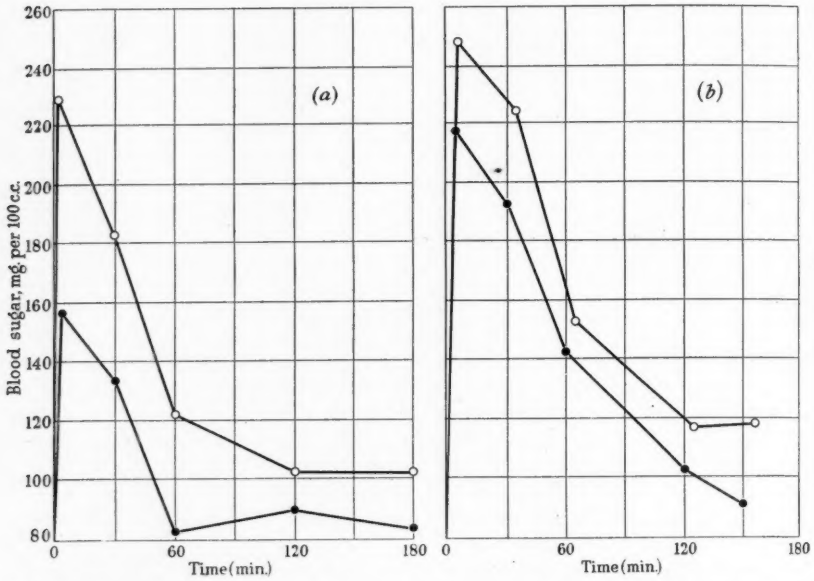

FIG. 1.—Glucose tolerance tests on rabbits. ○——○ = (*a*) 56, (*b*) 68 days after removal of right suprarenal. ●——● = 1 day after removal of left (remaining) suprarenal in each case.

TABLE II.—*Fermentable Carbohydrate Formation from Sodium* d-*lactate by Slices of Liver.*

0·5 g. slices shaken 1 hour at 38° C. in Krebs medium containing 0·45 per cent. neutralized *d*-lactate. Bicarbonate buffer. 95 per cent. O_2/5 per cent. CO_2 gas phase.

Normal.			Suprarenalectomized.			
Date.	Increase.		Date.	Days after removal of second suprarenal.	Increase.	
	mg./g. liver.	mg./kilo body weight.			mg./g. liver.	mg./kilo body weight.
16.x.39	2·18	52	12.x.39	7	3·10	86
27.x.39	7·67	188	13.xii.39	35	2·70	82
9.i.40	1·50	34	31.i.40	9	1·20	40
21.ii.40	1·16	29	15.iii.40	8	4·90	124
1.iv.40	3·22	94	6.iii.40	16	0·41	14
3.iv.40	5·10	133	16.ii.40	14	0·66	22
10.iv.40	2·94	73	27.iv.40	4	3·35	87
19.iv.40	3·59	84	18.v.40	5	5·54	104
9.v.40	1·60	49				

diminished, though not completely abolished in all cases. This point is illustrated in Table I. The glycogen formation is measured by the difference between the glycogen content of two batches of slices, one shaken in "plain" medium, the other in medium containing 1 per cent. glucose. For the experiment 0·5 g. of liver slices were used. The results are expressed as mg. of glycogen per kilo of body weight, as well as per g. of liver, since, as explained previously (Dawson and Holmes, 1939), the ratio liver weight/body weight is apt to vary considerably from one animal to another. On the other hand, we again verified that 48 hours' toxaemia certainly did not abolish the power of the liver to form glycogen from added glucose, except in two instances when the animals were in a collapsed condition and on the point of death when killed. Britton and Silvette (1934) have shown that suprarenalectomy diminishes the power of the liver in the living animal to deposit glycogen (Table I).

It has been shown that the power of the toxaemic rabbit to synthesize carbohydrate from lactic acid, pyruvic acid and alanine is very markedly reduced after 48 hours' toxaemia. A few experiments to test the power of liver slices from rabbits killed 24 hours after removal of the second adrenal to synthesize fermentable carbohydrate from lactate gave equivocal results. If, however, the animals survive for a week or more after the removal of the second gland, the liver seems to form fermentable carbohydrate from lactate quite as well as do livers from normal animals. This is illustrated in Table II.

It has previously been shown (Ostern, Herbert and Holmes, 1939) that the liver brei obtained from normal starved rabbits gives rise to the rapid phosphorylation of glycogen. Unless the concentration of glycogen is very high compared with that of phosphate, no phosphate disappears, because the glucose-1-phosphate which is formed is very rapidly broken down to glucose and phosphate. It has been suggested that this process is the most important pathway of breakdown of liver glycogen. If fluoride is added to the brei, as well as glycogen and phosphate, inorganic phsophate disappears because, although the hexose phosphate is formed, it can no longer break down again in the presence of fluoride. The rate of disappearance of phosphate under these conditions is thus a measure of the power of the liver to esterify glycogen, and probably therefore to convert it into blood sugar.

Table III shows that phosphorylation takes place both immediately and at longer periods after the removal of the second suprarenal. The process seems perhaps to go rather more slowly after removal of the second suprarenal, but the difference is slight, and many more experiments would be required to determine whether it is truly significant.

Diphtheritic toxaemia, in common with some other "toxic" conditions (for discussion see Holmes, 1939), markedly diminishes the power of the tissues to remove lactic acid. This is manifested in an accumulation of lactic acid in the blood, a slowing in the rate of disappearance of injected lactate from the blood (Dawson and Holmes, 1939; Hartmann and Senn, 1932; Stoffer, Dantes, Newburger and Sobotka, 1937; Stoffer, Dantes and Sobotka, 1938), and a diminution in the power of isolated liver tissue to dispose of added lactate (Cross and Holmes, 1937; Dawson and Holmes, 1939).

The figures obtained by Cross and Holmes, who shook liver slices for two

TABLE III.—*Phosphorylation of Glycogen.*

Liver brei suspended in an equal volume of fluid containing 10 mg. inorganic P (Sørensen buffer pH 7·3), 50 mg. glycogen, *M*/200 NaF. Allowed to stand, with stirring, at room temperature.

Normal.			Suprarenalectomized.		
Date.	mg. inorganic P disappearing per kilo body weight after—		Date.	mg. inorganic P disappearing per kilo body weight after—	
	30 min.	60 min.		30 min.	60 min.
18.iii.40	12·9	22·6	15.iii.40	8·9	18·4
8.iii.40	8·7	15·2	16.ii.40	19·4	29
27.iii.40	12·9	22·2	27.iv.40	11·8	12·4
10.iv.40	16	25·5			
Expetiment as above, but 200 mg. glycogen added.					
25.iv.40	—	12·0			
9.v.40	19·4	33·5	30.v.40	14·5	19·90
Average of 6 other experiments at various dates	20·8	29·5			

hours in medium containing 0·2 per cent. racemic lactate, gave a rate of removal of 1·18 mg. per g. tissue per hour for normal animals and 0·16 mg. per g. per hour for *48-hour* toxaemic animals. The contrast between the normal and toxaemic animals was very obvious. Dawson and Holmes repeated the experiments, but used more suitable conditions, i.e. 0·4 per cent. *d*-lactate, an experimental period of one hour instead of two, and smaller quantities of tissue. They obtained a far higher rate of lactate removal, which they expressed in mg. per kilo of body weight. Their figures were: normal, 233 mg./kilo. body weight/hour; *24-hour* toxaemic, 145 mg./kilo body weight/hour. The data for Cross and Holmes's experiments, when calculated on this basis, amount approximately to: normal, 62 mg./kilo body weight/hour; 48 hour toxaemic, 11 mg./kilo body weight/hour. The discrepancy between the two sets of experiments was at first thought to be explained by the fact that Cross and Holmes's data were obtained under conditions (i.e. the longer experimental time, larger amount of tissue, and lower concentration of racemic lactate) which were not optimal; but recent experiments performed in the same way as those of Dawson and Holmes have also given figures lower than theirs. The probable explanation is that there is a seasonal variation in the power of the rabbit liver to deal with lactic acid, and no doubt also in its other metabolic activities, since the experiments of Dawson and Holmes were all done in the early summer, those of Cross and Holmes in the late autumn, and the present series in the winter and early spring. The results of the present series are given in Table IV.

The results of this work seem to show quite clearly that, so far as the metabolic effects investigated are concerned, the picture seen in the tissues of a suprarenalectomized animal is quite different from that seen in those of an

TABLE IV.—*Removal of Lactic Acid by Liver Slices.*

0·4 g. of slices shaken for 1 hour at 38° C. in 3 c.c. Krebs medium containing 0·45 per cent. (neutralized) *d*-lactic acid. Bicarbonate buffer. 95 per cent. O_2/5 per cent. CO_2 in gas phase.

Normal animals.			Animals seven days or more after removal of second suprarenal.		
Date.	mg./g. liver.	mg./kilo body weight.	Date.	mg./g. liver.	mg./kilo body weight.
21.ii.40	6·17	154	6.iii.40	4·17	146
29.ii.40	6·96	167	16.ii.40	0·35	11·2
20.iii.40	10·55	259	..	..	..
18.iii.40	2·2	64	27.iv.40	11·44	296
1.iv.40	11·73	340	..	..	..
3.iv.40	14·67	384	18.v.40	4·36	82
10.iv.40	6·84	170	30.v.40	7·34	174

animal suffering from diphtheritic toxaemia. Since the whole adrenal was removed, our animals lacked both adrenalin and the cortical hormone or hormones. It thus seems unlikely that the disturbances of carbohydrate metabolism met with in diphtheritic toxaemia can be referred to suprarenal damage. They seem more likely to be due to damage to the liver cells themselves.

We do not, on the other hand, maintain that suprarenal damage plays no part in the picture of toxaemia, nor even that it may not be the cause of death, the disturbances of carbohydrate metabolism being superadded. We have, on the contrary, gained the impression that in the absence of the suprarenal animals are peculiarly susceptible to effects of infections, since we have lost several rabbits after the second adrenal had been removed. These animals suffered from slight sepsis at the site of the operation wound. In more than one case, all that could be seen was a small encapsuled abscess, and in no case was there peritonitis. In two cases attempts were made to save such animals by injections of cortical hormone. These attempts failed, and the animals were killed when moribund. In both instances, synthesis of glycogen and of carbohydrate proceeded normally, suggesting that the injected hormone had restored the metabolic picture to normal, though it had failed to save the animal's life. The relationship between surgical shock and the terminal condition in diphtheritic and other toxaemias has not yet been clarified. It is of interest that Swingle and Parkins (1935, 1938) claim that adrenalectomized animals are specially sensitive to shock.

SUMMARY.

Experiments have been made to test whether the known effects of toxaemia upon carbohydrate metabolism can be due primarily to damage by the toxin to the suprarenal glands. The livers of completely suprarenalectomized

rabbits form carbohydrate from lactate in a normal manner, and cause the disappearance of added lactate. They also phosphorylate glycogen, but form little glycogen from added glucose. Since it has previously been shown that in toxaemia gluconeogenesis is markedly diminished, lactate is only slowly removed, but glycogen is easily synthesized from added glucose, these experiments do not suggest that the disturbances caused by toxaemia are primarily due to damage either to the cortex or to the medulla of the suprarenal gland.

No alteration was observed in the glucose tolerance curve of suprarenalectomized rabbits.

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OBSERVATIONS ON LYMPHOCYTES IN TISSUE CULTURE.

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In general there are two schools of thought concerning the function of the lymphocyte. The first regard the lymphocyte as a multipotential or "embryonic" cell, and one consequence of their view is the acceptance of a unitarian theory of blood histogenesis. The second school deny that the lymphocyte possesses developmental potencies, and regard it as a mature and fully differentiated cell.

One of the most important problems of lymphocyte behaviour thus centres round the question of whether or not the lymphocyte is capable of undergoing transformations into other cell types. Most of the work which has been done on this problem by tissue culture methods has been carried out on material which already contains not only the cells into which the lymphocytes are alleged to develop, but also other cells (e.g. monocytes) which may themselves have the same developmental potency. In view of the difficulties of distinguishing between cell types, which are particularly acute in the study of living cells, the only really convincing evidence for such transformations would be in the form of serial photographs of the same cell at successive developmental stages. Deductions from observations on fixed and stained material, which mainly turn on the description of transitional types, are likely to be misleading.

The main evidence for the transformation of the lymphocytes into polyblasts and other cell forms was produced by Maximow (1916, 1917, 1922, 1923, 1924, 1927*a* and *b*, 1928). He worked with impure culture material of considerable variety and relied on interpreting "transitional forms" in fixed specimens. Awrorow and Timofejewskij (1914), Timofejewskij and Benewolenskaja (1926, 1927, 1928), Caffier (1928), de Haan (1928), Kreyberg (1929), Katzenstein (1931), Latta and Johnson (1934) all supported Maximow's view. Though Parker and Rhoads (1928*a*, *b*) and M. R. Lewis (1925) are sometimes quoted as in agreement with Maximow this is unjustified, as these authors only produced evidence that the monocyte underwent development. Bloom (1928, 1937) used the purest culture material obtainable, thoracic duct lymph of the rabbit, which he considered contained 99 per cent. lymphocytes. He fully supported Maximow.

On the other hand Shiomi (1925), Hirschfeld (1927), Veratti (1928), Seeman (1930), Wertheman (1932), Reeves (1934), Ehrich (1934), Murakami (1936) and Hall and Furth (1938) did not agree that the lymphocyte had developmental

potentialities. Some have explanted the buffy coat of the blood of rabbits and cockerels (Carrel and Ebeling, 1922; Vasiliu and Stoica, 1929; Jacoby, 1939). Macrophage-like cells developed which were derived from mononuclear leucocytes. None of these authors regarded it as definite that the lymphocyte took part in these transformations.

There is thus a conflict of evidence from observations on tissue cultures regarding the growth capacities of lymphocytes. In the following work Bloom's experiments, with certain slight modifications, have been repeated.

EXPERIMENTAL.

Preparation of Material.

Certain divergencies from Bloom's (1928) technique have been necessary. Pannet and Compton's (1924) saline was substituted for the Ringer solution used by Bloom. This is a phosphate-buffered saline solution having a pH of 7·5 when made up according to the author's description. This saline has been used instead of serum for the extraction of tissue. It is not clear why Bloom used serum instead of saline as an extracting agent, nor did he claim any specific advantage for it. Maximow (1928) has, in any case, recorded transformations in saline extracts.

Tissue extract.

Of the wide variety of tissue-extracts used by Bloom the two which he considered to favour the transformation of lymphocytes most strongly have been used, viz. marrow-extract and embryo-extract. Owing to the unavailability of rabbit embryos it was necessary to substitute chicken for rabbit embryo extract in all cases but one. It is not known whether rabbit embryo extract contains any factors which influence the growth of explanted tissue and which are not present in chicken extracts. According to Katzenstein, the presence of an extract is not necessary to obtain transformations.

Plasma.

Blood from rabbits was sometimes drawn from the heart or aorta into an ice-cold syringe containing 1 c.c. 1/1000 heparin for 20 c.c. blood. Owing to the frequency of spontaneous clotting, probably due to contamination with tissue juices, it was found more satisfactory to cannulate the carotid artery with a cannula moistened with liquid paraffin and to collect the plasma in ice-cold waxed tubes containing a similar proportion of heparin. The animals used for plasma had been starved for 24 hours before the operation and the plasma itself was generally used on the day of collection. Heparin (Heparin-Roche 318) was made up in 1 : 1000 solution in normal saline and autoclaved in sealed Jena glass ampoules.

Marrow extract.

This was aseptically removed from the femora and tibiae of the rabbits used for withdrawal of blood. After grinding, it was extracted in Pannet

and Compton's solution. The supernatant fluid was carefully examined for cells, and recentrifuged if necessary until none could be detected.

Lymph.

Preliminary experiments showed that Bloom's simple puncture of the ligated thoracic duct was unsatisfactory and that contamination was likely to result from it. A continuous flow of lymph was obtained from the indirectly cannulated duct (Sanders, Florey and Barnes, 1940). Samples were pipetted into small corked tubes where they were left to clot. Small pieces of the clot were used as explants.

Preparation of Cultures.

The clotting time and type of clot varied greatly in samples from different animals ; the best results were obtained when a loose clot was quickly formed. The concentration of lymphocytes in the lymph varied between 8000 and 78,000 per c.mm., but no correlation was established between concentration and subsequent behaviour.

The lymph clot was minced into fragments varying between 0·5 and 1·0 c.mm., and these were explanted into media consisting of one drop of plasma and one drop of either of the tissue extracts. To some of the cultures there were added, at a distance of about 0·5 cm. from the lymph explant, small pieces of 10-day embryonic heart tissue. The cultures were incubated as lying drops in sealed hollow ground slides. Bloom must be supposed to have used rabbit embryonic tissue, although he did not specify the source ; but on only one occasion was this possible in the present experiments. Since no appreciable difference was found between plain lymph cultures and those with the added tissue fragment, no distinction will be made between these sub-groups. The majority of cultures containing the lymph explant alone were immune from the possibility of infection with foreign cells from the tissue explant.

Rather more than 500 cultures were made in the manner which has just been described.

Controls.

Controls were made by one method or another for every series of cutures by one of the following methods :

1. Wet smears of thoracic duct lymph, homogeneous with the culture material, fixed in methyl alcohol or Zenker formol and stained with weak Delafield's haematoxylin and eosin. This was the routine stain ; satisfactory smears by the long Giemsa method were found to fade too quickly.

2. Sections of cultures prepared in precisely the same way as for experimental cultures (including explantation into the culture medium) but not incubated.

3. In some of the earlier experiments, sections of 2-hour cultures—i.e. before even the earliest claim for transformation.

Preparation of Sections.

Fixation and staining was carried out according to Bloom, who followed Maximow's (1909) directions. Briefly, fixation in warm Zenker formol was

followed by washing, dehydration and embedding in celloidin. Sections (7–10μ) were stained in weak Delafield and eosin or Weigert's haematoxylin and eosin.

The following estimate of the constitution of thoracic duct lymph has been obtained from determinations on wet stained smears: Large lymphocytes 6·6 to 24 per cent., small lymphocytes 63 to 87 per cent., monocytes 0·6 to 2·3 per cent., red blood cells 0 to 5 per cent. (the latter in one instance only) (Fig. 2).

Below are given other estimates of the constitution of thoracic duct lymph:

Author.	Method.	Monocytes (per cent.).	Lymphocytes (per cent.).		
			Large.	Medium.	Small.
Hall and Furth (1938)	Supravital	0·3	1·1	7·8	90·7
Murakami (1936)	Giemsa dry smear	0·7	16	..	82·3
Kindwall (1927)	Supravital	0·03	0·8	10	88
Murray, Webb and Swann (1926) (one estimate)	Unstated	5	30	..	65
Sanders (1939)	Supravital	1·3	4·3	..	94·4

The discrepancies are not surprising when one considers that there are at least seven different characters which afford a basis for distinction, and that since each character varies within a wide range, the system of classification of cells must still be largely a matter of personal choice.

RESULTS.

Observations on Living Cultures.

From the first hour of incubation onwards the cultures were kept under observation with an oil-immersion lens in an incubator box. Observations on individual cells could be made when the lymphocytes had migrated from the coagulum into the medium. Bloom's description of this as a "yellowish slightly opaque clot" was found to be just, and it is therefore not clear how he can claim to have made critical observations of the cells in this environment. Neither enlargement of the lymphocytes within the first 6 to 8 hours nor the subsequent development of fibroblast cells could be observed either in the explant or in the cells which migrated from it. The migration continued up to 48 hours but by 24 hours many were already dead (Fig. 1). The amount of migration varied greatly even in cultures of the same series, and was almost certainly due to variations in the size and consistency of the explants themselves. This inconstancy of behaviour among apparently identical cultures has been noted by Maximow, Bloom, Caffier and others. It is not likely to be due to small variations in the constitution of the medium since control sets showed an equally unpredictable behaviour.

Observations on Cultures Irrigated with Neutral Red.

Bloom treated his cultures with a "weak" solution of neutral red "for a short time." In our experiments, the exposure of the culture for three minutes

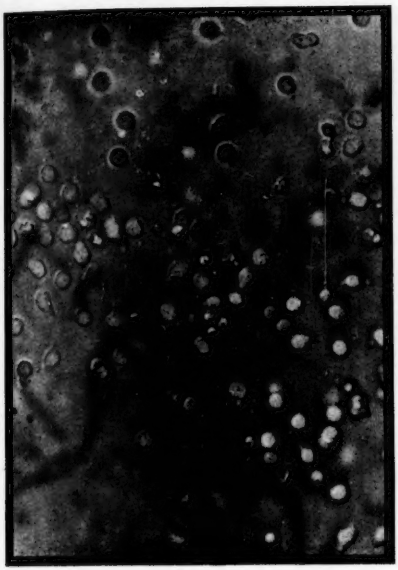

FIG. 1.

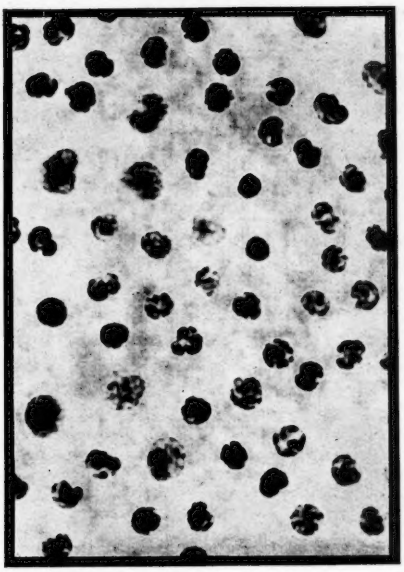

FIG. 2.

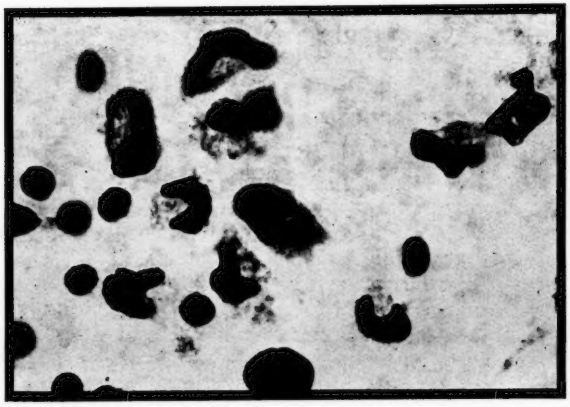

FIG. 3.

FIG. 1.—Part of migration field from lymph alone. 48 hours. Living preparation × 500.

FIG. 2.—Control smear of thoracic duct lymph, showing large and small lymphocytes. × 1000. (Methyl alcohol and Delafield's haematoxylin.)

FIG. 3.—Culture of rabbit lymph (6½ hours) from a hole in the thoracic duct. Monocytes, macrophages, large and small lymphocytes are present. × 1000. (Zenker-formol and Delafield's haematoxylin.)

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to a 1/15,000 to 1/20,000 solution of neutral red (Grübler) in saline was found to be satisfactory.

Within an hour from the time of application many of the lymphocytes contained one or two small neutral red droplets, and by about 6 hours the number had increased to 5 or 6. By this time 1 per cent. or less of the cells appeared under the low power as bright red dots and were found to contain very much more neutral red than the others. The droplets almost filled the cell and obscured the nucleus. It was found impossible to decide whether they should be classified as monocytes or as large lymphocytes but, in all cultures treated with neutral red, a fairly constant proportion could be counted. Very slow movements could occasionally be seen in these larger cells. After 24 hours observations on these living cultures became extremely difficult, and it is not clear how, in using this (Bloom's) technique, the optical properties of the clot could be improved upon.

Sectioned and Stained Material.

Cells corresponding to the figures of polyblasts in Maximow and Bloom's *Text-book of Histology* (p.145) have been found in 5, 7, 9 and 21-hour cultures and in one case in a 3-hour culture. Typical small macrophages, not seen in the controls, have also been found at the 6th hour. Differential counts of control cultures and of cultures at 6, 9 and 21 hours showed that the number of cells which could be described as hypertrophied lymphocytes, polyblasts or macrophages (Bloom's terminology) was largest in the neighbourhood of the 6th hour.

On the basis of differential counts similar to these Hall and Furth (1938) concluded that these cells had been developed from monocytes. In a population not originally homogeneous and whose constituents are multiplying at different rates, such a conclusion must be accepted with caution.

Cultures of Lymph Collected by Bloom's Method.

One culture series was prepared from the lymph of an animal which had died three minutes previously. In this case the lymph was collected from a hole in the thoracic duct, i.e. by Bloom's method, and not from a cannula. It is for this reason that it must be considered more fully. The lymph was macroscopically free from blood. A rough supra-vital count gave a figure of 5 per cent. large cells, which could not be classified with certainty into large lymphocytes and monocytes. A differential count of 500 cells on a Delafield-eosin smear, in which it was also impossible to distinguish between the large cells, gave the following percentages: Large cells, 23; small lymphocytes, 63·4; pseudo-eosinophils, 11·8; erythrocytes, 1·8. Control sections, however, gave a figure of at least 5 per cent. monocytes, but the cells were too tightly packed for accurate counting. About 4 monocytes in mitosis were counted in 400 cells.

This lymph clotted very poorly and many of the explants were no more than clumps of cells loosely held together by strands of fibrin. As might be expected, migration from the lymph clots was extensive. At 6 hours the following cell-types could be distinguished: small and large lymphocytes, a

few dead lymphocytes, pseudo-eosinophils, many monocytes and large hypertrophied monocytes (macrophages = polyblasts (Bloom)) (Fig. 3). Most of the pseudo-eosinophils were dead after 24 hours and the lymphocytes gradually degenerated, until by the 4th day there remained only phagocytic cells about twice the size of a large lymphocyte. Most of these were dead by the 5th day, no doubt because they were not subcultured ; but a few were still moving and a mitotic figure was found in one preparation. The changes we have described occurred in only a few cultures of this series.

DISCUSSION.

Previous evidence makes it quite clear that there develop in vitro from lymph, blood and lymphatic tissue, cells which are morphologically distinct from any type originally present and which behave in a different way. It is furthermore generally agreed that these cells do not arise from granulocytes but from the non-granular cells of the blood or from some younger precursor. The majority of haematologists now agree that the monocyte of the blood is capable of developing into a cell indistinguishable from a tissue macrophage. On the subject of the developmental potencies of the lymphocyte opinions are, however, divided.

The repetition of Bloom's work which this paper described did not confirm his conclusions. The points raised by the single culture series in which the lymph was not taken from a cannula but, in the manner of Bloom, from a hole in the thoracic duct, are very suggestive. It was only in this culture series, use being made of a manifest imperfection in technique, that results comparable to those of Bloom were recorded ; and it raises the question of whether the high initial percentage of monocytes was due to this method of collection—the only one which Bloom believed to be practicable. It is true that in this instance the abdomen had been massaged before the collection of lymph ; but this was not found, in another experiment, to alter the concentration of monocytes in lymph flowing from a cannulated duct. It is therefore possible to correlate the large number of phagocytes which appeared in cultures of this particular series with the high initial monocyte concentration of the explants.

The Distinction between Large Lymphocytes and Monocytes.

Unfortunately the distinction between large lymphocytes and monocytes is so fine that many authors obviously use both terms to describe what may be a single species of monunuclear non-granular cell. It will be remembered that Seeman (1930) in his cultures of lymph nodes and blood recorded transformations not only in monocytes but in cells which he calls " monocytoids." As far as can be judged from his descriptions, Maximow would have called these cells large lymphocytes or transitional forms. Ehrich (1934) does not exclude the possibility that large lymphocytes undergo further developmental changes, because, as he says, it is not always possible to distinguish between large lymphocytes, monoblasts and lymphoblasts.

The most important consequence of this discussion is that since, as has been abundantly proved, the monocyte is capable of undergoing development into

another type of cell, then it cannot always be confidently denied that the large lymphocyte does so also. But the evidence for transformations of small lymphocytes is not satisfactory.

SUMMARY.

In the majority of cultures of rabbit lymph collected through a cannula lymphocytes were found to persist unchanged. In others there appeared cells which Bloom would have described as polyblasts, but whose origin could not be definitely determined.

In one instance the cultivation of lymph collected from a hole in the thoracic duct yielded results comparable with those of Bloom. These results it is suggested were correlated with the contamination of the original source of lymph, made possible by Bloom's method.

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OBSERVATIONS ON LYMPHOCYTES IN CHAMBERS IN THE RABBIT'S EAR.

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THE results of observations by tissue culture methods which have been recorded in the previous paper were disappointing in that they failed to substantiate the theory of the developmental potentialities of lymphocytes (Medawar, 1940). Successful observations had been made by Ebert and Florey (1939) on the transformation of monocytes into histiocytes using a modification of the Sandison-Clark chamber in the rabbit's ear. It appeared to us that the best way of settling whether lymphocytes turn into polyblasts, macrophages and fibroblasts was to observe by similar methods such a change actually occurring in vivo. Observations on lymphocytes have, however, been much more difficult to make than on monocytes.

Clark, Clark and Rex (1936) have described the appearance of lymphocytes in amphibia and rabbit's-ear chambers as follows: "The lymphocytes in the tissue move with their relatively large compact nucleus in advance and a small cytoplasmic process extending posteriorly. Their behaviour outside the vessels is characterized by alternating periods of rapid migration in a relatively straight course followed by longer periods of inactivity in which they assume a rounded form." And again they made the following observations in a rabbit's-ear chamber: "A few typical lymphocytes were also present in this region on this day. They were easily distinguishable from polymorphs by their smaller size, lack of granules, less amount of cytoplasm and characteristic movement." Movements of lymphocytes have also been observed in tissue culture by Lewis and Webster (1921). The appearance of the lymphocytes and their type of movement observed in our chambers correspond in every way with the description given by these authors, so that it is not proposed to give a further lengthy description here. McCutcheon (1924) described and measured the rate of locomotion of lymphocytes from human blood. He found that in the course of some hours all lymphocytes became motile.

EXPERIMENTAL.

During the course of some hot summer days of 1939 lymphocytes were recognized in our rabbit's-ear chambers (Ebert, Florey and Pullinger, 1939) by reason of their size and the type of motion already mentioned.

It quickly became apparent that the temperature of the ear had a considerable influence on their motility. A heating apparatus was therefore devised so that the chamber was kept at about

37° C. It consists of two rectangular brass boxes, placed on the stage, one on each side of the rabbit's ear. The boxes each contain two 12v. "Trafficator" bulbs wired in parallel. The boxes are pivoted about two brass pillars screwed to the moving stage and can be locked in any position. To the top of each box is fastened a brass plate arranged to overlap its fellow of the opposite side; a portion of each is cut away so as to clear the objective. The lower end of the space enclosed by the boxes is filled with a strip of brass between the pillars. All odd crevices are loosely packed

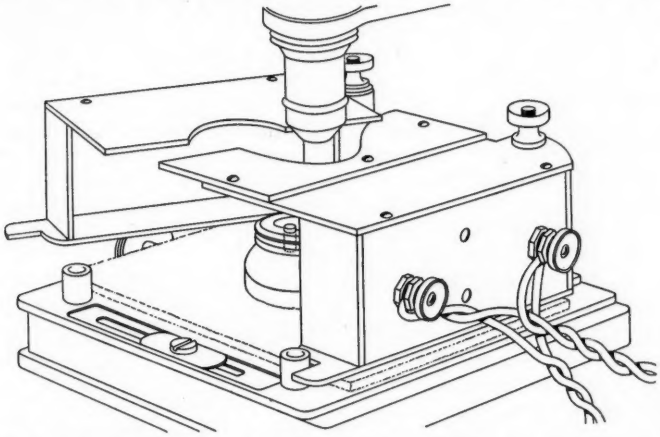


FIG. 1.—Heating apparatus for warming rabbit's ear on stage of the microscope.

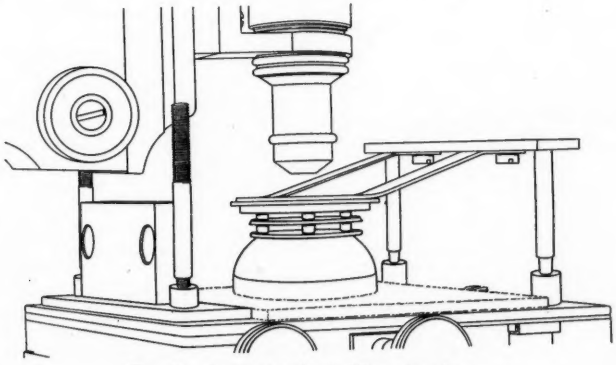


FIG. 2.—Apparatus for holding chamber.

with cotton-wool. A thermometer is inserted when needed. The temperature is controlled by a variable resistance (Fig. 1).

For holding the chamber down on the bakelite dome previously described (Ebert and Florey, 1939), a special clip has been devised which greatly facilitates the procedure and lessens the chance of damaging the delicate tissue in the chamber. A brass ring with three recessed holes fits over the top of the chamber and rests, not on the cover slip ring, but on the silver bolts. A second ring, fitting over this and rotating freely on it, bears two lateral wings. On these wings pressure is exerted by two phosphor bronze strip springs borne on a brass bar above the rabbit's ear and plugged into the moving stage like an ordinary stage clip (Fig. 2).

The lymphocytes were encountered both in the tissue and in the space between the tissue and the mica coverslip. They frequently adhered to the mica. The number of lymphocytes seen varied greatly with the condition of the chamber. It was quite unusual to find any in a fully vascularized old chamber with much fibrous tissue present, but they could be seen while the tissue was still growing. In some chambers small areas of chronic infection which became surrounded by cells of various types were particularly favourable for finding lymphocytes. It was thought at first that observations on a lymphocyte or group of lymphocytes could be made at intervals and their changes, if any, noted. We were, however, soon disillusioned, for the lymphocyte can move so rapidly that nothing short of constant observation is sufficient to keep a given lymphocyte under observation for long periods of time. To facilitate following a given moving lymphocyte one eye-piece of the binocular microscope was furnished with a hair, the tip of which was in focus and was at the centre of the field. The mechanical stage could then be manipulated to keep the observed lymphocyte near the tip of the hair.

The reasons for regarding the cells we have watched as lymphocytes are as follows: (1) *Size:* when quiescent and spherical their diameter was about 6 to 7·5μ. (2) *Method of locomotion:* the characteristic feature of lymphocyte movement in vitro is that the nucleus appears to travel foremost followed by a small tail of protoplasm. This type of movement was described by Clark, Clark and Rex in living tissue and was constantly shown by the cells we have observed. (3) Using a special type of injection chamber Janus green solution was applied to some living cells. The mitochondria of those of the size of lymphocytes stained well to produce a picture typical of the arrangement in the lymphocyte.

For injecting dyes or other fluids into the tissue on the table two types of chambers have been made, called, for convenience, the "screw-on" and the "built-in" injectors. In both types access to the tissue is through a 1 mm. hole, normally closed by a perspex plug, similar to that described already (Ebert, Florey and Pullinger, 1939). In the "screw-on" injector the silver bolts project through the base of the chamber and carry, when the injection is to be made, a second base plate bearing a small projecting silver tube which enters the hole in the chamber. This silver tube conveys the dye from a "duct" drilled in the detachable base to the chamber. The "duct" opens at the edge of the base and is large enough to take the nozzle of a micro-injection apparatus. When the base and injection apparatus have been fitted, the chamber is placed on the stage and the injection made under visual control. The entire operation is done aseptically (Fig. 3).

In the second type or "built-in" injector the duct is permanently in the base of the chamber, which has to be thicker than usual. To prevent tissue growing down the hole from the table a long plug of perspex is kept there until the injection is to be made, when a short plug is substituted. One advantage of the built-in type is that it is easier to have two access holes to the table, one for the dye and the other for a transplant. Double injector chambers of both types with inflow and outflow holes have also been constructed and have been used successfully (Fig. 4).

Since the lymphocyte cannot be "marked" with carbon as can the monocyte, nor coloured with vital dyes for any length of time, it was necessary to carry out all observations at one session. The first observations were made for about two hours on unanaesthetized rabbits, but for more prolonged work some form of anaesthesia was necessary. At first intravenous nembutal was used, but the relatively short duration of anaesthesia resulted in a restless animal. Intraperitoneal injection of urethane was found satisfactory. This

drug (1·5 gm. per kilo) produced satisfactory narcosis for 24 hours or longer. At the end of the experiment large quantities of normal saline were given subcutem to hasten elimination of the drug and recovery of the animal. The presence of anaesthetic introduces a complication in that it might be argued that anaesthesia prevents transformation of cells. No difference whatever has been noted in the lymphocytic movements in anaesthetized and non-anaesthetized animals. In the present work only small lymphocytes have been observed, so that any possible confusion with monocytes has been avoided.

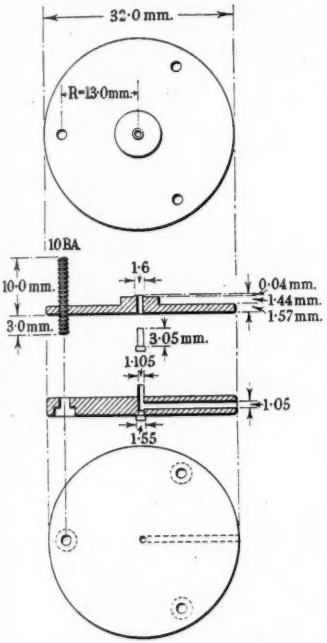


FIG. 3.—" Screw-on " injector chamber.

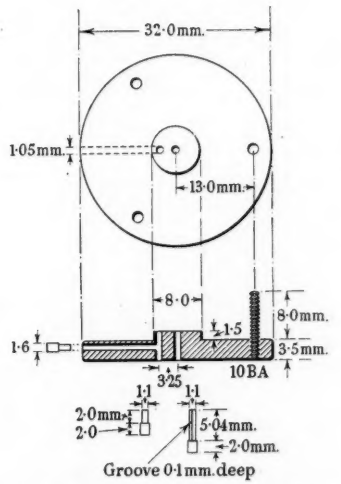


FIG. 4.—" Built-in " injector chamber.

In addition to the observations of lymphocytes " naturally " occurring in certain chambers we have transplanted fragments of popliteal lymph glands into chambers. In the first experiment of this type a small amount of lymph gland was pushed into a central transplant hole (see Ebert, Florey and Pullinger (1939) for type of chamber), so that numbers of lymphocytes were forced between the mica coverslip and the underlying vascularized tissue. For a further elaboration of this procedure a special chamber was constructed (see Fig. 5). This chamber was made so that the newly formed tissue over the central table was 250μ thick and therefore richly vascularized. By means of the double lid it was possible to lift only the mica coverslip, and place a small fragment of the rabbit's own popliteal gland (taken aseptically) directly on the newly

vascularized tissue. A new mica coverslip was then applied and observations carried out on the lymphocytes which emerged from the transplant. An attempt was made to separate the transplanted lymph gland from the vascularized tissue by means of a very permeable " gradocol " membrane so that no cells

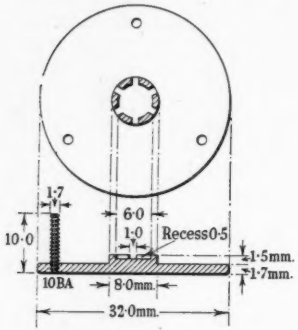
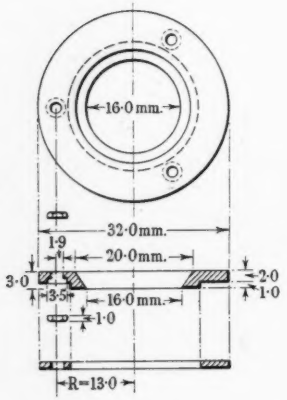


FIG. 5.



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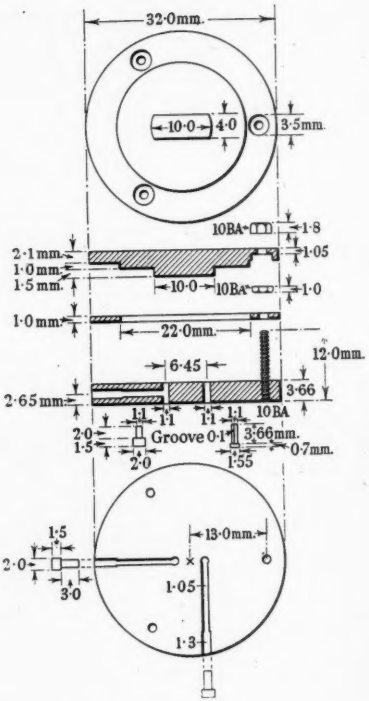


FIG. 6.

FIG. 5.—Base plate of chamber for transplantation of lymph gland.
FIG. 6.—" Lacune " chamber.
FIG. 7.—Top plate or " coverslip " for chamber of type of Fig. 6. This is applied after the removal of the solid perspex coverslip. The washers separating the base plate and coverslip have been omitted in all drawings.

from the tissue could wander into the transplant, but this was not successful owing probably to too low an oxygen tension above the membrane. The lymphocyte appears to be rather sensitive to oxygen lack.

A further modification of the chamber which may be of considerable use in studies on tissue cells was also developed. Unfortunately, owing to prevailing conditions, few observations have been made with it, but we feel that it may be of use to describe it now. It was noticed frequently

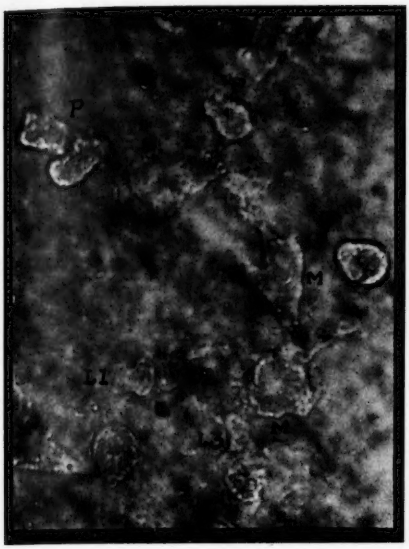
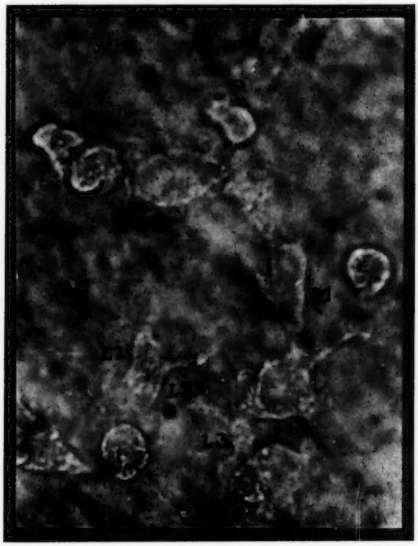


FIG. 8.



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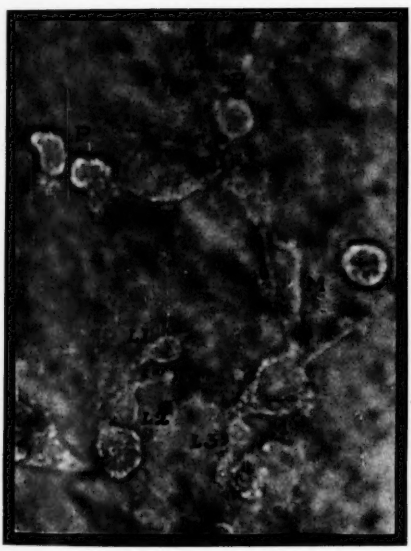

FIG. 10.

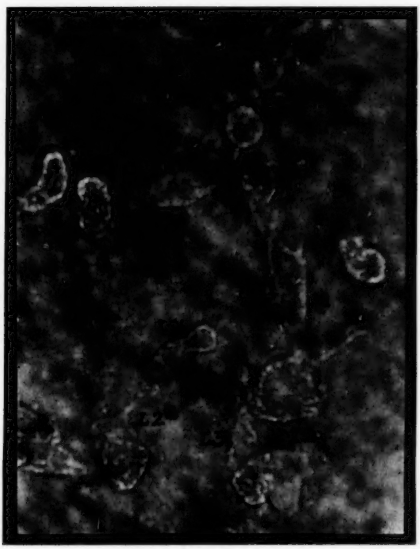

FIG. 11.

FIG. 8.—Two lymphocytes, L1 and L2, in contact with one another. Another lymphocyte, L3, is also present.

FIG. 9.—One minute after Fig. 8, the lymphocytes are moving in opposite directions.

FIG. 10.—One minute after Fig. 9.

FIG. 11.—One minute after Fig. 10. Note "tails" of lymphocytes.

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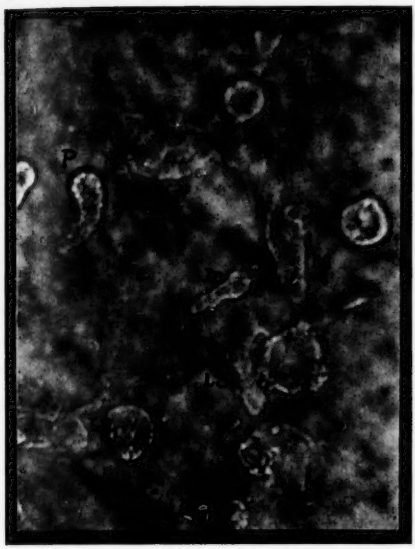

Fig. 12.

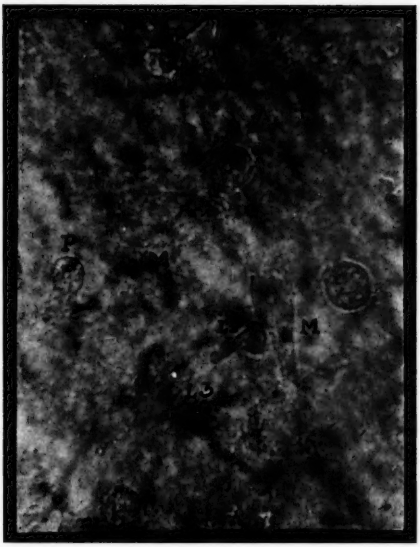

Fig. 13.

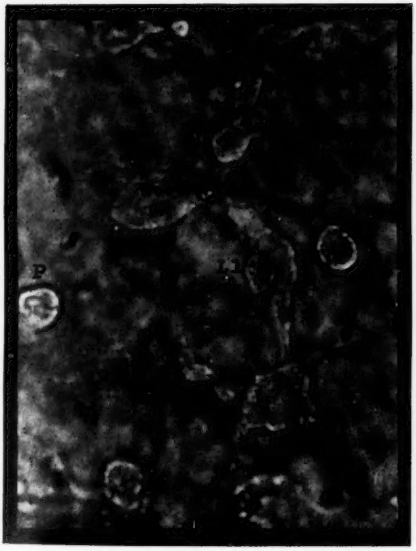

Fig. 14.

Fig. 15.

Fig. 12.—Two minutes after Fig. 11.
Fig. 13.—One minute after Fig. 12.
Fig. 14.—Three minutes after Fig. 13.
Fig. 15.—Two and a half minutes after Fig. 14.

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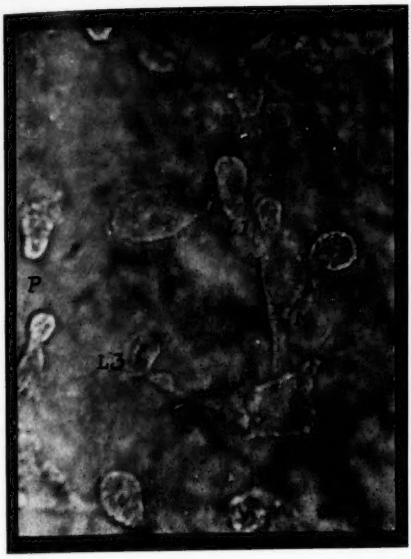

FIG. 16.

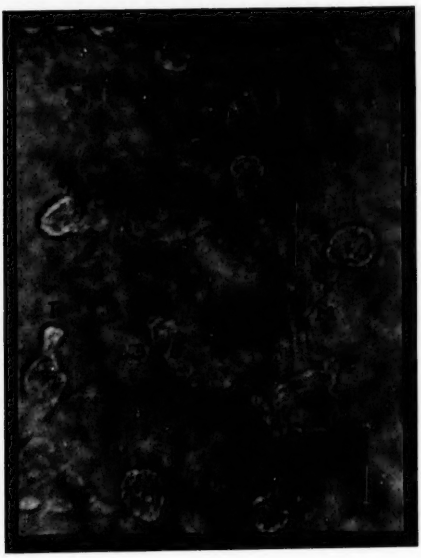

FIG. 17.

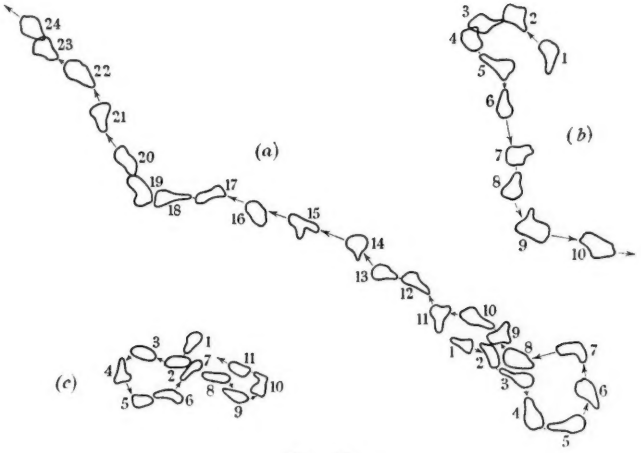

FIG. 18.

FIG. 16.—One minute after Fig. 15.

FIG. 17.—One minute after Fig. 16. In Figs. 8 to 17 some polymorphs are marked P and some macrophages M.

FIG. 18.—(*a*) Camera lucida record of the path of a lymphocyte during one hour. Positions plotted at approximately two-minute intervals. (*b*) Path of a lymphocyte traced for 21 minutes. (*c*) Path of a lymphocyte traced for 33 minutes.

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that "lacunes" of crescentic shape developed at the sides of the central table when chambers were replaced (see Ebert and Florey, 1939). The lacunes were filled with transparent fluid and cells of many types—in particular macrophages—attached themselves to the mica and made admirable objects for microscopical study. The disadvantage of the lacune however was that it was at the extreme edge of the microscopic field. A chamber was therefore made without a central table (see Fig. 6). A coverslip made entirely of perspex had a central depressed rectangular portion which, when the chamber was screwed in position, touched the base plate. A hole in the cartilage of the rabbit's ear was cut to accommodate this rectangle. The ear was allowed to heal and then the perspex coverslip removed and replaced by a mica-carrying coverslip (Fig. 7). A central space in the ear was thus left which filled with fluid. For a few days large numbers of cells were present, but soon the lacune became clear and the cells adherent to the mica could be admirably seen. By incorporating injection ducts in the base plate it was possible to inject into the lacune or space dyes or any other fluid. In the course of time the lacune gradually became smaller and eventually was obliterated by tissue, but many weeks were available for observation purposes.

RESULTS.

It may be stated at once that we have observed no transformation of lymphocytes into other cells even when the observations have been unremittingly carried on for many hours by observers taking "shifts." The transformations described are said to occur between 8 and 14 hours. It should be emphasized that the lymphocytes observed were already present in the tissue or on the mica coverslip when the observations began. That is to say they must have migrated from the blood vessels at some indeterminate time beforehand. Careful observations were made on the state of the blood vessels to determine if active inflammation was present. Almost without exception not even polymorphs were adhering, and it is therefore probable that the lymphocytes observed in the tissues had been there for a considerable time. It was noted that the prolonged exposure to light did not cause inflammation. It may be objected that the necessary stimulus to change was not present in our chambers. This however is unlikely, as many of the observations were carried out on lymphocytes at the edge of a chronic infection. Many macrophages of all sizes were present, so there was little doubt that the stimulus for the formation of macrophages existed. In one observation the lymphocyte apparently died after 15 hours. It was not at once ingested by a nearby macrophage, but after about 7 more hours it was definitely inside this cell, and was partially digested in the next 5 hours. Hydrokollag—a graphite suspension—was injected in one experiment using an injection-type chamber in which many lymphocytes were present. Though the numerous macrophages present rapidly took up the particles, no attempt at phagocytosis was noted in lymphocytes although they were actively motile.

Clark, Clark and Rex (1936) state that they did not observe lymphocytes in lymphatics, but that the "small round cells" were polymorphs undergoing some degenerative change. We have, however, in two animals observed many cells in lymphatics which moved about in the lymphatic in typical lymphocyte fashion.

The observations on the transplanted lymph glands were of considerable interest. Live motile lymphocytes were found in the neighbourhood of blood vessels, in one case—a thin chamber—for 7 days; in another they were observed to be numerous during 8 days, at the end of which time the graft had almost

disappeared. In another case many lymphocytes were still present after 26 days, when observations were discontinued. The graft at this time had practically disappeared, though its site could still be recognized by the presence of many vessels and macrophages.

It has become necessary to abandon the work on these grafts on account of the war. This is unfortunate, for the preparation offers admirable opportunities for observations of lymphocytes.

It must be pointed out that prolongation of observation much beyond 24 hours does not seem very possible with this technique. To watch a single cell a team of workers is required to take turns at the microscope, and more important, rabbits do not stand anaesthesia well for a longer period.

The macrophages showed only very sluggish movements compared with lymphocytes and polymorphs. It was only possible to detect changes in shape by observations over several minutes and then they were slight. Their surfaces, however, frequently had projecting from them fine hair-like protoplasmic processes. These were also very clearly seen on giant cells.

The accompanying figures illustrate some of the observations (Figs. 8 to 18).

SUMMARY.

These observations show that small lymphocytes are very actively motile cells in chambers in the rabbit's ear. They are often quite as active as polymorphs, though the most active polymorphs move more rapidly.

The macrophage type of cell changes its shape and position relatively very slowly. The macrophages exist in a great variety of shapes and sizes. They display fine hair-like protoplasmic protuberances in many cases.

No evidence has been obtained of small lymphocytes turning into other cells. Individual lymphocytes have been followed up to 24 hours. These observations do not exclude changes taking place in the course of days.

Several new types of chamber are described which facilitate in vivo cellular observations.

All the chambers have been made in the laboratory by Mr. S. W. Bush, to whose great skill we are much indebted. Mr. J. D. Kent has had charge of the animals. The diagrams are by Mr. H. Axtell.

We are indebted to the Medical Research Council for a grant towards the expenses.

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THE ENZYMIC ACTIVITY OF VACCINIAL ELEMENTARY BODIES.

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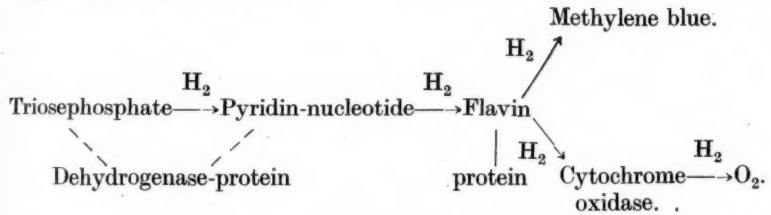
A PRELIMINARY investigation of the enzymic activity of suspensions of the elementary bodies of vaccinia virus was reported by Macfarlane and Salaman (1938); all the preparations tested contained a phosphomonoesterase and catalase, but showed no dehydrogenase activity towards any of the substrates tested. It is known that the biological oxidation or oxido-reduction of a substance involves the presence not only of an enzyme, a dehydrogenase, activating this particular substance, but of linked enzyme systems by means of which hydrogen is transferred from the substance through a series of hydrogen-carrying coenzymes or prosthetic groups to its final acceptor, which may be either oxygen or some other metabolite (see Dixon, 1939). The type of enzyme system required may be exemplified for the oxidation of a particular substrate, triosephosphate, as follows:



It is clear that the dehydrogenase activity of an organism or an isolated enzyme cannot be demonstrated *in vitro* by the reduction of a dye or the uptake of oxygen unless the hydrogen-carrying system is complete.

The previous tests on the dehydrogenase activity of the virus were carried out on the working hypothesis that, though the number of dehydrogenases present might be very few, the hydrogen-carrying system would be complete and the activity as readily demonstrable as that of a well-washed bacterial suspension: that is to say it was assumed that the physical and chemical constitution of the virus was essentially similar to that of micro-organisms. The view was put forward recently by McFarlane *et al.* (1939) that it is more in keeping with the physical and chemical properties of the virus to postulate for it a rigid or semi-rigid structure akin to a giant protein molecule, than a structure limited by a semi-permeable membrane and functioning on osmotic

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principles. The negative results of the tests previously made for dehydrogenase activity in the virus might have been due to the removal of the dissociable coenzymes from the virus, in absence of a semi-permeable membrane, by the repeated washing necessary in the preparation of the suspensions. The tests have therefore been repeated in the presence of the appropriate coenzyme and of a suitable flavoprotein and have again given negative results. Since further work on this subject has been suspended a brief report of these and other experiments on the enzymic activity of the virus is given.

EXPERIMENTAL.

Vaccinia virus.

Homogeneous suspensions of elementary bodies in *M*/250 citrate-phosphate buffer, pH 7·2, were prepared as previously described (Salaman, 1937), the strain of virus having now been passaged more than 100 times on rabbit's skin. A part of each preparation was used as the inoculum for the next lot of rabbits ; all the preparations were thus known to be highly infective.

Tests for Enzymes.

Dehydrogenases.

The coenzymes used were pure specimens of di- and tri-phosphopyridinnucleotides and alloxazine-adeninedinucleotide. As flavoprotein either " coenzyme factor " prepared according to Straub (1939) or a specimen of Warburg's " yellow enzyme " were used. For positive controls preparations of triosephosphate mutase and lactic dehydrogenase were made from rabbit skeletal muscle (Green *et al.*, 1937 ; Green and Brosteaux, 1936) ; comparative experiments were made with suspensions of well-washed *Bact. coli*. Triosephosphate was prepared according to Meyerhof (1938), the preparation containing 33 per cent. of the total P as phosphoglyceraldehyde. The protocol of a typical experiment with the Thunberg technique using as substrate triosephosphate is given (Table I). Similar tests were carried out either by the Thunberg or

TABLE I.—*Triosephosphate Dehydrogenase.*

The complete system contained : 1 ml. virus suspension (1·6 mg.) or 1 ml. *Bact. coli* (2·4 mg.) or 1 ml. muscle extract ; 1 ml. coenzyme factor or yellow enzyme ; 0·1 mg. diphosphopyridinnucleotide ; 0·5 ml. 0·02 *M* triosephosphate, pH 6·8 ; 0·5 ml. 0·15 *M* phosphate buffer, pH 6·8 ; 0·2 ml. 0·0005 *M* methylene blue ; total volume to 3·3 ml. with water.

Enzyme.	Coenzyme factor.	Yellow enzyme.	Reduction time. (min.).
—	+	—	>180
—	—	+	>180
Muscle extract	—	—	>180
,, ,,	+	—	4
,, ,,	—	+	105
Vaccinia virus	+	—	>180
,, ,,	—	+	>180
Bact. coli	—	—	3

+ = present ; — = absent.

manometric techniques using (1) lactate or boiled liver extract as substrate, in presence of diphosphopyridinnucleotide and coenzyme factor ; (2) hexosemonophosphate with triphosphopyridinnucleotide and yellow enzyme ; (3) *dl*-alanine with alloxazine-adeninedinucleotide. Tests were also made with preparations of vaccinia virus which had been dried and extracted with ether or alcohol-ether. All these tests were negative and are therefore not reported in detail. Similar tests were made with specimens of tobacco mosaic virus and Bushy stunt virus, using triosephosphate and lactate as substrate, and were also negative.

Zymohexase and enolase.

The vaccinia virus suspensions were tested for the presence of zymohexase, which converts hexosediphosphate into triosephosphate and can therefore be recognized by the production of alkali-labile P, and for the presence of enolase and phosphoglyceromutase, which convert 3-phosphoglyceric acid into phosphopyruvic acid, a change recognized by the increased rate of hydrolysis of phosphate in acid solution. Tables II and III show that none of these enzymes was present in the virus suspensions, or in the final washings of the virus, though they were easily detected in the tissue extract from which the virus had been first deposited.

TABLE II.—*Zymohexase Activity.*

3·0 ml. 0·05 *M* Na hexosediphosphate ; 1·0 ml. virus suspension (2·2 mg.) or 1·0 ml. tissue extract ; pH 7·3 ; 37° C.

	Mg. alkali-labile P.	
	20 sec.	120 min.
Substrate alone	0·03	0·11
,, + vaccinia virus . . .	0·04	0·09
,, + virus washings . . .	0·07	0·11
,, + tissue extract . . .	0·09	0·77

TABLE III.—*Enolase Activity.*

3·0 ml. 0·1 *M* Na 3-phosphoglycerate ; 1·0 ml. virus suspension or tissue extract ; pH 7·2 ; 37° C.

	Mg. P hydrolysed in 30 min. in *N* HCl at 100° C.	
	Zero.	After 60 min. incubation.
Vaccinia virus	0·01	0·01
Tissue extract	0·03	0·93

Carbohydrases.

No increase in reducing power was observed after incubation of vaccinia virus with α-methyl glucoside, maltose, cellobiose, lactose or saccharose for 24 hours at 37° C. The virus also had no hydrolytic action on adenosine.

Peptidase.

The hydrolytic action of the vaccinia virus on *dl*-leucylglycine in veronal buffer at pH 7·6 was investigated by titration of amino groups in presence of formaldehyde. Though a small increase in amino groups was detected, the very slow rate of hydrolysis, with the consequent difficulty of excluding contaminating or autolytic action and the experimental error involved in the titration in presence of protein, made these experiments inconclusive.

Phosphodiesterase and polynucleotidase.

Preliminary experiments showed that the virus suspensions could hydrolyse diphenylphosphate and therefore contained a phosphodiesterase (Table IV); enzymes of this type can hydrolyse the di-ester type of linkage postulated as that between nucleotides in nucleic acids. In investigating the action of the virus on nucleic acids special precautions were taken against bacterial growth, because of the slow rate of hydrolysis of these substances. All the solutions and glassware were sterilized. The virus suspension was treated with ether for 1–3 days at 0° C., the virus deposited by centrifuging immediately before use, washed with *M*/100 KCl in order to remove any soluble enzymes liberated by autolysis and resuspended in *M*/100 KCl. Since bacterial counts made after 48 hours' incubation of test mixtures showed that even so growth might occur, all further experiments were made in the presence of chloroform, which ensured sterility without appreciably affecting the nuclease activity.

TABLE IV.—*Phosphodiesterase Activity.*

1·0 ml. 0·01 *M* K diphenylphosphate ; 1·0 ml. 0·06 *M* veronal-HCl buffer ; 1·0 ml. 0·01 *M* $MgSO_4$; 0·5 ml. virus suspension (2 mg.) ; water to 3·75 ml. 1 drop $CHCl_3$; pH 8·6 ; 37° C.

	Mg. phenol in total volume after (hr.)	
	24.	70.
Substrate + buffer	·002	·002
Virus + ,, ...	·007	·007
,, + substrate	·036	·095

The hydrolytic action of the virus suspensions on samples of B.D.H. thymo- and ribonucleic acids, which had been purified by repeated solution in alkali and reprecipitation by acid, was tested in acetate, citrate or veronal buffer at pH 6·6, 7·8 and 8·4, in the presence of 0·006 *M* $MgCl_2$. The method used was essentially that of Macfadyan (1934). The enzyme action in the test mixture (1·5 ml.) was stopped, and the unchanged nucleic acid precipitated, by the addition of 1·0 ml. of 1·25 per cent. uranyl acetate in 10 per cent. trichloroacetic acid. The precipitate was deposited and washed twice with 2 ml. diluted (4 : 10) uranyl acetate reagent, dissolved in 2 ml. 5 per cent. Na_2CO_3 and made up to 10 ml. The amount of nucleic acid was determined by colorimetric phosphate estimations after ashing. The combined supernatant and washings were also made up to 10 ml., and the amount of the hydrolysed nucleic acid present as nucleotide and as inorganic phosphate determined by estimation of the total and inorganic phosphate ; these values have been omitted from the Tables as they have no great significance.

Table V shows the hydrolysis of ribonucleic acid by vaccinia virus. It will be seen that the rate of hydrolysis both of diphenylphosphate and of ribonucleic acid was much slower than that of the phosphomonoesters (*cf.* Table VI); this relationship is generally observed also in tissue extracts (see Levene and Dillon, 1932). Thymonucleic acid was only very slowly attacked by the virus suspensions (Table V).

TABLE V.—*Polynucleotidase Activity.*

0·5 ml. Na nucleate; 0·5 ml. buffer; 0·5 ml. virus or bacterial suspension; 37° C.

(A) *Ribonucleate.*

Enzyme.	Buffer.	pH.	$CHCl_3$.	Mg. P as nucleic acid after (hr.). 0.	22.	48.	99.
None	Citrate	6·6	—	1·09	1·11	..	1·11
Virus, B125, 2 mg.	,,	6·6	—	1·12	0·91	..	0·56
Ditto	Veronal	7·8	—	1·12	0·92	..	0·72
,,	,,	8·4	—	1·12	0·94	..	0·83
None	Citrate	6·6	—	0·82	..	0·82	..
Virus, B126, 2 mg.	,,	6·6	—	0·80	..	0·55	..
Ditto	,,	6·6	+	0·80	..	0·55	..
S. aureus 0·8 mg.	Acetate	6·6	+	0·80	..	0·81	..
,, 1×10^8/ml.	,,	6·6	+	0·80	..	0·80	..
,, ,,	,,	6·6	—	0·80	..	0·79	..

(B) *Thymonucleate.*

None	Citrate	6·6	—	1·18	1·18	..	1·19
Virus, B125	,,	6·6	—	1·19	1·18	..	1·05
,,	Veronal	7·8	—	1·19	1·17	..	1·15
Ditto	,,	8·4	—	1·19	1·17	..	1·15

Phosphomonoesterases.

The rates of hydrolysis of adenosine-3-phosphate (yeast adenylic acid), adenosine-5-phosphate (muscle adenylic acid) and β-glycerophosphate by vaccinia virus suspensions have been compared. Table VI shows that

TABLE VI.—*Phosphomonoesterase Activity.*

0·5 ml. ·05 *M* substrate; 0·5 ml. buffer, containing 0·01 *M* $MgCl_2$; 0·5 ml. virus or bacterial suspension or water; 2 drops $CHCl_3$.

Enzyme.	pH.	Mg. inorganic P after 2 hrs. at 37° C. with Adenosine-5-phosphate.	Adenosine-3-phosphate.	β-glycerophosphate.
None	6·6	·007	·005	·005
Virus (2 mg.)	6·6	·101	·010	·014
S. aureus (0·9 mg.)	6·6	·005	·007	·005
None	8·4	·007	·007	·005
Virus (2 mg.)	8·4	·180	·021	·072
S. aureus (0·9 mg.)	8·4	·007	·007	·005

adenosine-5-phosphate was very much more rapidly hydrolysed than the other esters ; this fact is of interest in view of the existence of a specific 5-nucleotidase in many tissues (Reis, 1937) and the possible participation of this enzyme in the hydrolysis of nucleic acid (Gulland and Jackson), 1938.

Phosphatase activity of Staphylococcus pyogenes aureus.

A washed suspension of this organism, which is a constant bacterial contaminant of the fresh virus suspensions, was prepared from a 24-hours' broth culture. The hydrolysis of nucleic acid and phosphomonoesters by the bacteria, in amounts of the order of 100 to 10,000-fold that of the bacterial content of the virus suspensions before treatment with ether, was almost negligible (Tables V and VI), whether chloroform was present or not. It seems probable that this is not due to a lack of phosphatases in the bacteria, but to the limited access of the substrates to the enzymes within the intact cell. It may be noted that the presence of phosphatases in bacteria was demonstrated by Pett and Wynne (1938) using dried organisms. The low activity of the *Staph. aureus* suspensions indicates the extreme unlikelihood that the nuclease activity of the virus suspensions was due to contamination with bacterial enzymes.

Phosphatase Activity of Plant Viruses.

Two samples of Bushy stunt virus and the single sample of tobacco mosaic virus examined showed phosphatase activity towards disodium phenylphosphate in the pH range 5 to 7, though the activity was low compared with that of the vaccinia virus in the optimum pH range 8 to 9 (see Table VII). A third sample of Bushy stunt virus was inactive. The infectivity of these viruses was not determined at the time the phosphatase experiments were made.

TABLE VII.—*Hydrolysis of Disodiumphenylphosphate by Plant Viruses at* 37° *C.*

		Mg. phenol liberated in 6 hrs. at pH—				
		8·4.	7·0.	6·0.	5·4.	4·8.
Bushy stunt 1st sample,	0·4 mg.	0	..	·02	..	..
,, ,, 2nd ,,	0·7 ,,	..	..	·024	·011	·003
,, ,, 3rd ,,	0·5 ,,	..	0	0	..	..
Tobacco mosaic virus	0·5 ,,	..	·022	·024	..	..

DISCUSSION.

It is hardly credible that the phosphatase activity which has been observed is due to any impurity in the virus suspensions when other enzymes, easily detectable and of equally wide distribution in animal tissues, such as zymohexase, nucleosidase and certain dehydrogenases, are absent. At the same time the reasonable conclusion that the phosphatase activity is inherent in the virus throws into sharp relief the absence of enzymes which play an important part in the energy-producing activities of cells. It may be inferred that if the virus possesses enzymes which can synthesize protein or carbohydrate or nucleic acid, it can probably only exercise these functions by stealing energy from the host. The mechanism by which the energy obtained by the oxidation of foodstuffs by certain cell-enzymes is made available for the synthetic action of other

enzymes is very obscure ; it seems quite possible that a virus protein lying within the cell may be in as good a position to absorb this energy as the enzymic proteins of the cell.

The significance of the presence of phosphatases can be considered from two aspects, the hydrolytic and the synthetic. The hydrolytic activity is of interest in the pathology of the virus ; it may clearly be of primary importance in the degeneration of an infected cell whose normal metabolism is bound up with the presence of nucleoprotein, phospholipin and phosphoric esters. In this connection it is of particular interest that the virus rapidly hydrolyses adenosine-5-phosphate, which is an essential coenzyme in the metabolism of carbohydrate by many types of cells. It may be presumed that phosphomono- and di-esterases intervene in the synthesis of nucleic acids and other phosphoric esters, and from this aspect it is remarkable that although the vaccinia virus contains a nucleic acid of the desoxypentose type (McFarlane *et al.*, 1939), it hydrolysed ribonucleic acid much more rapidly than thymonucleic acid. The samples of the latter used for the test may have been in a highly aggregated form (Schmidt *et al.*, 1938), and if such aggregation takes place through the phosphoric acid groups, a very much larger number of linkages must be broken before the appearance of the soluble nucleotides by which the hydrolysis was measured. The possibility may also be mentioned that the relative rates of hydrolysis and synthesis by the nuclease may differ with ribo- and thymonucleic acids as in the case of papain, which hydrolyses hippuryl amide but synthesizes, in similar experimental conditions, hippuryl anilide from hippuric acid and aniline (Bergmann and Fraenkel-Conrat, 1937).

Though the presence of phosphatases may undoubtedly be of significance in the synthesis of any nucleoprotein, the problem in the case of a reproductive nucleoprotein is that of the synthesis not only of nucleic acid and protein but of phosphatases and proteinases. The striking analogy drawn by Northrop (1937, 1939) between the multiplication of viruses and the autocatalytic formation of certain proteolytic enzymes from inactive precursors of similar molecular weight has the drawback that it relegates the problem of synthesis of the virus to that of the synthesis of a precursor. Though proteins of high molecular weight have been isolated from normal plants susceptible to the plant viruses, the weight of chemical and serological evidence is against the hypothesis that they are precursors of the viruses (Bawden and Pirie, 1938). The formation of the proteolytic enzymes by autocatalysis is a special case of a general reaction, for they can be formed from their precursors not only by other organic but by inorganic catalysts, e.g. the formation of trypsin by the action of $MgSO_4$ on trypsinogen. It has been shown that chicken pepsin and swine pepsin are serologically distinct, and that the product of the action of chicken pepsin on swine pepsinogen is not chicken pepsin but swine pepsin (Herriott *et al.*, 1938). The latter phenomenon has been regarded as an " adaptation " of the enzyme analogous to the adaptation of a virus, but it may also be interpreted as a primary catalytic action of chicken pepsin followed by the autocatalytic action of the swine pepsin so formed. It seems clear that in these cases the serological species specificity is latent in the precursor, and is revealed in the enzyme independently of the agent by which the hydrolytic formation of the latter takes place. But if plants of different species, e.g. tobacco and tomato, are

infected with the same virus, or if plants of the same species are infected with different viruses, the virus recovered is apparently serologically identical with that used as inoculum ; it seems probable that here the serological specificity is due to the virus which must therefore have some capacity to synthesize.

It is difficult to visualize a protein particle containing nucleic acid or carbohydrate or lipin which could synthesize these various substances and incorporate them in a replica by the exercise of only one specific enzymic activity. It seems more probable that such a particle would be a community of different enzymes which are arranged in a fixed relationship to each other, and can reproduce the whole by the concerted exercise of their individual specific activity. It may be recalled that phosphatase has been demonstrated in a strain of bacteriophage (Schüler, 1935) as well as in vaccinia virus, and the few experiments we have made suggest that this enzyme is also present in certain plant viruses. Further work on the enzymic activity of different viruses is clearly desirable in regard to the question of their reproduction. The parasitism of a virus may possibly be referred to a lack of oxidizing enzymes, while that of man, who is dependent on other organisms for vitamins necessary for his oxidative processes, must be considered to be due to a lack of enzymes capable of synthesizing these substances. From this point of view the transformation of a medium into virus or cell or tissue will depend on the variety and pattern of enzymes in the primary community, and not on any fundamental difference between the behaviour of a protein unit in a virus particle and that of a protein unit in a cell.

SUMMARY.

Suspensions of the elementary bodies of vaccinia virus have been tested for dehydrogenase activity towards lactate, triosephosphate, hexosemonophosphate and alanine in the presence of the appropriate coenzymes and flavoprotein, with negative results. The suspensions also lacked zymohexase, enolase, α- and β-glucosidases and nucleosidase. The elementary bodies contained phosphomono- and di-esterases and hydrolysed ribonucleic acid ; the rapid rate of hydrolysis of muscle adenylic acid is particularly interesting with regard to the pathology of the virus.

The authors wish to thank Dr. F. C. Bawden for specimens of the plant viruses, and Prof. O. Warburg for the gift of coenzymes.

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THE LATENT CARCINOGENIC ACTION OF 3 : 4-BENZPYRENE; RESULTS OF INTERMITTENT APPLICATIONS TO THE SKIN OF MICE.

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The rate of absorption and elimination of benzypyrene has been shown to be of great importance in determining the reaction of connective tissues to this substance after subcutaneous or intramuscular injection (Peacock and Beck, 1938). The incidence of sarcoma varied roughly in inverse ratio to the rate of absorption; continuous exposure for three to five months gave rise to sarcoma in the majority of mice.

When painted on the skin of mice in a volatile solvent, benzpyrene regularly induces carcinogenesis if the applications are repeated twice a week for three months, and it is customary in assaying a carcinogenic substance to apply it in this manner. Fluoroscopic observation shows that after applying a 0·3 per cent. solution of benzpyrene in benzene (violet fluorescence), the solvent rapidly evaporates, leaving solid benzpyrene (yellow fluorescence) in contact with the epidermis, but that solution then occurs progressively in the fats and lipoid substances present in the epidermis, so that the fluorescence again becomes violet within about 15 minutes of the application being made. After a single application, the skin remains fluorescent for about four days, and after penetration of the epidermis has begun, the hydrocarbon cannot be removed by washing with fat solvents; fluorescence diminishes progressively, however, as absorption and elimination proceed. Thus, in the case of benzpyrene applied twice a week in the manner described, the skin of the mouse is continuously exposed to the action of the hydrocarbon. The latent period in

experimental carcinogenesis varies considerably between different individuals and for different chemical carcinogens, and it is known that during this latent period irreversible changes precede the clinical appearance of a tumour (Guldberg, 1931 ; Orr, 1938). Whether complete recovery from subcarcinogenic doses is possible is not yet known. The minimum period of application of a chemical carcinogen that will lead to tumour development is not exactly known, but Bang (1922), and later, Leitch (1922), showed that tumours, varying in type between simple papilloma and carcinoma, developed on the skin of mice that had been painted with tar for four months, after which a latent period of variable length was noted before the development of the tumour.

Human cases of carcinoma have been reported following splashing of the skin or conjunctiva with hot tar or pitch, but such cases are undoubtedly rare (Gunsett, 1930).

In the following experiments an attempt has been made to find out, approximately, the limit of tolerance of mouse skin for benzpyrene, and to ascertain how far the effects of subcarcinogenic exposure are cumulative.

METHOD.

Seven groups of stock mice were painted with 0·3 per cent. solution of 3 : 4-benzpyrene in benzene, as detailed below :

Group 1 : Sixteen mice were painted twice a week for one month only.

Group 2 : Seventeen mice were similarly painted for two consecutive months only.

Group 3 : Twenty mice were similarly painted for three consecutive months only.

Group 4 : Nineteen mice were similarly painted during alternate months for a period of six months.

Group 5 : Nineteen mice were similarly painted for three separate monthly periods, separated by two-monthly intervals.

Group 6 : Nineteen mice were similarly painted for three separate monthly periods, separated by three-monthly intervals.

Group 7 : Nine mice were painted every 8th day for six months. This amounts to four-day periods of exposure to benzpyrene, separated by four-day recovery periods throughout the experimental period.

RESULTS.

Only those mice which survived for three months or more from the beginning of the experiment have been recorded, and the results are shown graphically in Figs. 1 to 7, the numbers of which correspond with the above groups. Each dotted line represents the life of an individual mouse ; full lines represent the growth of papillomata, and expanding lines the growth of carcinomata. All tumours, except those in animals still living, have been histologically confirmed.

It will be seen that the period of continuous action of benzpyrene that can be supported by the mouse epidermis without ultimate carcinogenesis lies

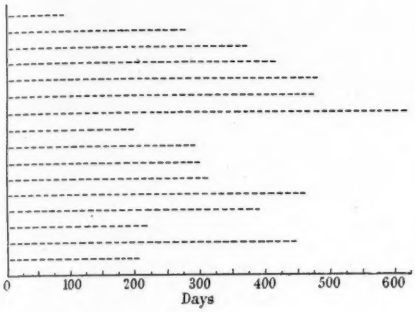


FIG. 1.

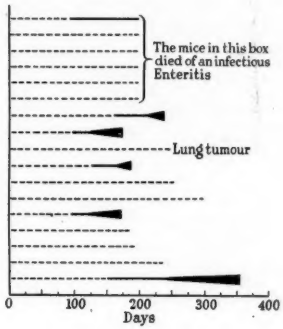
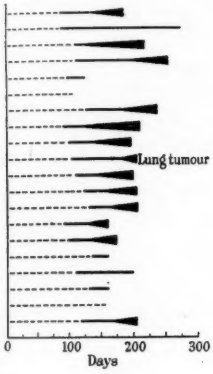


FIG. 2.



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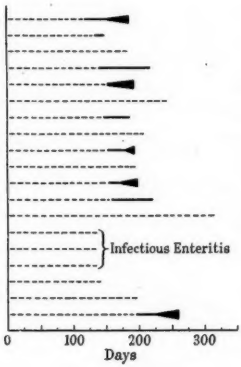

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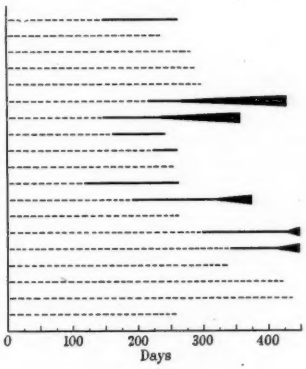

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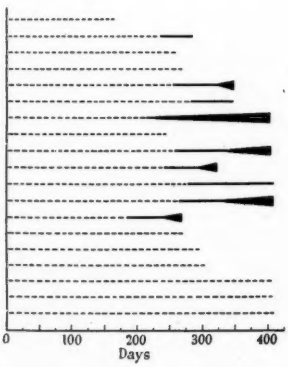

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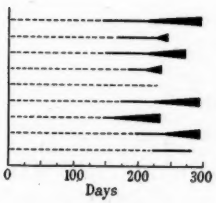

FIG. 7.

between one and two months (Figs. 1 and 2). In Groups 4 to 6 the introduction of intervals during which no benzpyrene was applied to the skin gave partial protection though the more susceptible animals developed tumours. In Group 7 there was no evidence of protection, and it is possible that the period of four days, during which fluorescence was not visible by direct observation of the animals in the ultra-violet beam, did not represent a true recovery period.

DISCUSSION.

These results emphasize the importance of recognizing that apparently normal skin may have been so modified by repeated exposure to carcinogens that a subsequent application, which would not damage normal skin, may provoke a carcinogenic response. In earlier experiments, when tar was the usual carcinogen employed, research workers could see at once if their hands remained contaminated after experimenting with this substance, but when dilute solutions of colourless carcinogenic hydrocarbons are used, no visible trace can be seen unless the hands, gloves and instruments are examined for fluorescence in the ultra-violet beam, as a routine. Immediate washing with ether soap solution within a few minutes of contamination removes almost all the hydrocarbon, but after a delay, even of 15 minutes, it is impossible to remove it completely.

SUMMARY.

0·3 per cent. solution of 3 : 4-benzpyrene in benzene was applied intermittently to the interscapular skin of stock mice, with the following results :

(1) Painting twice a week for one month does not induce tumour growth.

(2) Similar painting for two months induces papillomata and carcinomata in about a quarter of the animals that survive for three to four months.

(3) Intervals of from one to three months between three monthly periods of painting do not protect the skin completely, though tumours are less frequent than when the same amount of benzpyrene is applied without intervals for recovery.

(4) The skin of mice painted in the manner described remains fluorescent for about four days ; thus, twice weekly painting exposes the skin to the continuous action of benzpyrene.

(5) Painting every eight days for six months induces as many tumours as painting every four days for three months.

(6) The danger of repeated exposure of human skin to the action of dilute solutions of carcinogenic hydrocarbons is emphasized, and means of protection are advocated.

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THE SPONTANEOUS DISINTEGRATION OF CERTAIN BLOOD PIGMENTS, WITH SPECIAL REFERENCE TO METHAEMALBUMIN FORMATION.

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THAT haemoglobin is spontaneously converted into methaemoglobin has been recognized ever since Hoppe-Seyler (1865) named the latter pigment, and it has been universally agreed that haematin is a later disintegration product in the katabolism of the methaemoglobin.

Fairley and Bromfield (1934) demonstrated a new blood pigment in the plasma of blackwater fever cases. Owing to its general resemblance to methaemoglobin, Fairley (1937) named it pseudo-methaemoglobin, and the following year, when pseudo-methaemoglobin was synthesized from alkaline haematin (ferric) prepared from pure haemin and human crystalbumin, the pigment was re-named methaemalbumin by Fairley (1938).

Serial quantitative observations, based on the degree of dilution necessary for the extinction of the α bands of methaemalbumin and oxyhaemoglobin in plasma derived from blackwater fever patients, had indicated that methaemalbumin was derived from extracorpuscular oxyhaemoglobin. Considering this pigment to be in some way peculiar to blackwater fever, Fairley and Bromfield (1937) had postulated that the plasma in this disease might contain the agent responsible for its formation. When investigating this possibility, oxalated plasma was collected under sterile conditions from a patient who was recovering from a bout of blackwater fever; this plasma, which at that time contained no demonstrable new pigment, was then incubated with a concentrated solution of oxyhaemoglobin at 40° C. After 17½ hours traces of a pigment with an α band in the red resembling methaemoglobin appeared, and at 42 hours it was demonstrable in considerable quantities. On the Hartridge reversion spectroscope, however, the α band in the 42-hour specimen was not colinear with that of artificially prepared methaemoglobin, while its chemical behaviour differed inasmuch as Stokes's reagent (2 drops per c.c.) and 10 per cent. ammonium sulphide (1 drop per c.c.) failed to disperse the α band, which they do immediately with methaemoglobin solutions. Spectroscopically and by other tests, the pigment formed on incubation was identified as the same as that occurring in blackwater fever plasma. When the control system containing normal plasma and haemoglobin incubated under similar conditions was examined, it was unexpectedly found that the pigment with the α band observed in the red portion of the spectrum was not methaemoglobin as previous workers had thought, but the same as that found in blackwater fever, i.e. methaemalbumin.

The technique used and the actual experiments on which these and subsequent conclusions have been based have not previously been recorded, and in the present paper it is proposed to summarize these experimental findings.

I. TECHNIQUE AND METHODS EMPLOYED IN IDENTIFYING DIFFERENT BLOOD PIGMENTS.

(1) *Spectroscopy.*

A reversion spectroscope was used to determine the position of the bands of the different pigments under investigation. In the differentiation of methaemalbumin from methaemoglobin and sulphaemoglobin, the effects of different reagents like Stokes's reagent, 10 per cent. ammonium sulphide and concentrated ammonium sulphide on the α band in the red portion of the spectrum were investigated. The latter reagent has been widely employed in Schumm's test (1912) for the detection of haematin in plasma, a positive reaction being characterized by the appearance of a haemochromogen with a sharply defined α band at 558$\mu\mu$. The method now adopted is to cover a given volume of serum with ether and to run in with a pipette one-tenth the volume of concentrated ammonium sulphide, subsequently shaking. Since it is now known that haematin always combines with human serum albumin to form methaemalbumin, it follows that this reaction in human plasma signifies the presence of methaemalbumin and not free haematin as was previously thought.

(2) *Harrison's Apparatus.*

When traces of pigment were suspected, the fluid under investigation was examined by direct spectroscopy in Harrison's (1938) apparatus. This apparatus enables a considerable depth of fluid to be examined, and reveals small quantities of pigment the presence of which might otherwise remain undetected or doubtful.

(3) *pH Determination.*

In a limited number of experiments, determinations of the pH of the haemoglobin solution or haemoglobin-plasma mixture under investigation were made daily by Dr. J. C. Broom, using a glass-electrode pH metre.* The necessity for this was realized when it was found that the pH of the system increased on incubation at 37° or 40° C. from approximately 7·6–7·8 to 8·6–9·2 when serum or plasma was present. Hartridge (1920) found that at a pH of 8·0 neutral methaemoglobin began to be transformed into alkaline methaemoglobin, and that this transformation was complete at a pH of 9·0. In the presence of high concentrations of haemoglobin alkaline methaemoglobin is not readily identified, and as the transformation of neutral methaemoglobin into alkaline methaemoglobin leads to a disappearance of the α band in the red, the presence of methaemoglobin in the system may never be suspected unless the pH be reduced to the acid side of 8·0. When the pH lies between 8·0 and 9·0 a mixture of neutral and alkaline methaemoglobin is present ; in these circumstances the addition of *N* HCl (1 drop per 2 c.c.) or a few crystals of

* The apparatus, which makes use of a special spiral electrode, was designed by Dr. C. S. Pope of the Wellcome Physiological Research Laboratories, and is manufactured by Unicam Instruments Ltd., Cambridge.

potassium dihydrogen phosphate intensifies the α band of neutral methaemoglobin at 630μμ by converting alkaline into neutral methaemoglobin.

(4) *The Detection of Mixtures of Methaemoglobin and Methaemalbumin.*

Where there is a mixture of methaemalbumin and neutral methaemoglobin in plasma, the α absorption band lies somewhere between 623μμ and 630μμ according to the relative concentrations of the pigments present. If the pH of the plasma be changed to 9·0 or over by the addition of *N* NaOH (1 drop per 2 c.c.), any neutral methaemoglobin present is immediately converted into alkaline methaemoglobin ; then only the α band of methaemalbumin is found at 623μμ to 624μμ. A similar result may be obtained by the addition of 10 per cent. ammonium sulphide (1 drop per c.c.), which disperses the methaemoglobin and leaves the α band of methaemalbumin intact at 623μμ to 624μμ.

(5) *Concentration and Identification of Methaemalbumin in the Albumin Fraction.*

This is perhaps best illustrated by citing a typical experiment : A human plasma-haemoglobin mixture with a pH of 7·8 and containing 12 per cent. haemoglobin (Haldane) was incubated at 37° C. for 10 days. After this period the pH was 9·1 and alkaline methaemoglobin and methaemalbumin were found in high concentrations. Some 30 c.c. were taken and the albumin and globulin fractions separated by the ammonium sulphate method. The globulin precipitate when dissolved in water was brown in colour, and showed an α band at 630μμ discharged by 10 per cent. ammonium sulphide ; with concentrated ammonium sulphide no haemochromogen was formed, i.e. negative Schumm's test. These findings indicated that the globulin fraction contained methaemoglobin, but not methaemalbumin.

The albumin fraction was dialysed against running water for several days, then against distilled water and later phosphate buffer (pH 8·0). The resulting fluid was brown in colour with a pH of 7·76. It was too dense for spectroscopic examination, but after suitable dilution a high concentration of methaemalbumin but no traces of haemoglobin or methaemoglobin were found. The spectrum was typical of methaemalbumin, the well-defined α band being situated at 623μμ, a β band at 540μμ and a γ band about 500μμ.

Chemical reactions.

The usual chemical tests differentiating methaemalbumin from certain other blood pigments with an α band in the red portion of the spectrum were performed. The α band was not dispersed by Stokes's reagent (2 drops per c.c.), 10 per cent. ammonium sulphide (1 drop per c.c.) or hydrogen peroxide (10 volumes) ; it was dispersed, however, by concentrated ammonium sulphide (analar), and formed the typical haemochromogen with an α band at 558μμ.

Sodium hydrosulphite ($Na_2S_2O_4$).—On adding to the albumin fraction (pH 7·76) solid $Na_2S_2O_4$, the colour changed from brown to red and the typical two-banded spectrum immediately appeared. There was a prominent α band at 573μμ and a β band at 530μμ. The spectrum was typical of haemalbumin, containing ferrous iron, and differing quite definitely from the broad single band of reduced haematin. After a lapse of a few minutes the β band faded, and on addition of more $Na_2S_2O_4$ was replaced by a single broad hazy band at

about 574$\mu\mu$ which was difficult to measure. This now completely replaced the original two-banded spectrum and resembled reduced haematin. The solution was both acid in reaction (pH 5·1) and turbid, and on centrifuging all the pigment was found to have come down in the brown precipitate, the turbid supernatant fluid being quite colourless. The broad single band, which had only appeared after the original methaemalbumin had been destroyed, was probably reduced haematin split off from its albumen component.

Sodium hydrosulphite and carbon monoxide.—On treatment with $Na_2S_2O_4$ and CO a red solution was produced, which on spectroscopic examination showed a prominent α band at 571$\mu\mu$ and a β band at 534$\mu\mu$ resembling carboxyhaemoglobin.

Comment.

The spectroscopic picture and the chemical reactions given by this brown pigment were typical of methaemalbumin, which had been isolated in high concentration in the albumin fraction* free from methaemoglobin and haemoglobin.

II. INCUBATION EXPERIMENTS WITH HAEMOGLOBIN AND HUMAN PLASMA-HAEMOGLOBIN MIXTURES (37° C. AND 40° C.).

The serum or plasma used in these experiments has always been collected under sterile conditions, and as a routine, 4 volumes of human plasma have been incubated at 37° C. or 40° C. with 1 volume of a concentrated solution of oxyhaemoglobin prepared from freshly lysed human corpuscles. The final concentrations of haemoglobin in the system have generally varied from 10 to 15 per cent. (Haldane). As a control, an aqueous solution of haemoglobin prepared from well-washed human corpuscles lysed in distilled water was incubated at the same temperature and daily examination of the two systems were made. The findings in a typical experiment may be studied in Table I.

(1) *Haemoglobin.*—It will be observed that on incubation at 37° C. the pH of the haemoglobin solution shows no alkaline shift, and that neutral methaemoglobin appears within 20 hours and persists in increasing quantity throughout the experiment. The presence of haematin was not revealed spectroscopically and Schumm's test was negative over a period of 13 days, but owing to the presence of oxyhaemoglobin and methaemoglobin in quantity, such negative findings have doubtful significance. The pH, which was originally 7·26 in this experiment, had only increased to 7·76 during this long period of incubation.

(2) *Haemoglobin-plasma mixture.*—With the human plasma-haemoglobin mixture it will be noted that the pH soon changed to the alkaline side of 8·0. At the end of 20 hours, the pH had changed from 7·7 to 8·3 and a mixture of alkaline and neutral methaemoglobin was demonstrated. Spectroscopic examination showed a faint α band at 630$\mu\mu$, which became definitely intensified on the addition of 1 drop of *N* HCl. This was due to a reduction in pH resulting in the transformation of alkaline methaemoglobin into neutral methaemoglobin.

* The albumin fraction containing methaemalbumin has since been successfully dried *in vacuo* over sulphuric acid. The resulting brown solid residue contains methaemalbumin which is water-soluble and present in a relatively pure and stable form; it should now prove possible to purify the pigment further by separating the other proteins contained in the albumin fraction.

TABLE I.—*Incubation of Human Plasma and Haemoglobin* (37° C.).

Time.	System.	pH.	Methaemoglobin.		Methaem-albumin.	Schumm's test.
			Neutral.	Alkaline.		
Before incubation	Plasma + Hb	7·7	0	0	0	0
	Haemoglobin	7·26	0	0	0	0
20 hours	Plasma + Hb	8·3	+	+	0	0
	Haemoglobin	7·26	+	0	0	0
44 ,,	Plasma + Hb	8·6	+	+	+	+
	Haemoglobin	7·3	+	0	0	0
92 ,,	Plasma + Hb	8·6	+	+	+	+
	Haemoglobin	7·3	+	0	0	0
112 ,,	Plasma + Hb	8·6	+	+	+	+
	Haemoglobin	..	+	0	0	0
136 ,,	Plasma + Hb	8·6	±	±	++	+
	Haemoglobin	..	+	0	0	0
184 ,, or later	Plasma + Hb	8·9	0	0	++	++
	Haemoglobin	7·5	+	0	0	0

Final haemoglobin concentration = 15 per cent. (Haldane); pH = 7·8. ++ = much present or strongly positive; + = positive or present; ± = trace; 0 = nil or negative; Hb = haemoglobin.

At 44 hours the pH was 8·6 and a mixture of alkaline methaemoglobin, neutral methaemoglobin and methaemalbumin was demonstrated. Spectroscopic examination showed a well-defined broad band in the red at 625$\mu\mu$, which was replaced by a band at 623$\mu\mu$ on adding dilute ammonium sulphide (10 per cent.), i.e. methaemalbumin; this shift was due to the dispersal of neutral methaemoglobin, leaving the α band of methaemalbumin intact. Schumm's test was positive. On adding 1 drop *N* HCl to 2 c.c. of the plasma-haemoglobin mixture the α band appeared at 627$\mu\mu$, the shift being due to the formation of additional neutral methaemoglobin from the alkaline methaemoglobin.

Similar findings were recorded at 92 and 112 hours (Table I). At 136 hours the amounts of alkaline and neutral methaemoglobin had decreased, whereas the methaemalbumin had markedly increased; at 184 hours when the pH equalled 8·9, neutral and alkaline methaemoglobin were no longer detectable and much methaemalbumin was present. A strong α band was situated at 623$\mu\mu$ and remained unaltered by the addition of *N* HCl or dilute ammonium sulphide (10 per cent.). Schumm's test was strongly positive.

III. INCUBATION OF WHOLE BLOOD AT 37° C.

For this purpose sterile oxalated whole human blood was incubated at 37° C. with minimal disturbance of the two layers of plasma and corpuscles, all specimens being carefully pipetted off. On each occasion the corpuscles were washed in saline and lysed in distilled water before being examined spectroscopically. The results are incorporated in Table II.

It will be noted that the pH of the lysed corpuscles was 7·56 prior to incubation and 7·3 some 137 hours later, whereas the pH of the plasma was 7·86 prior to incubation and 8·9 after 137 hours' incubation. The plasma contained small quantities of oxyhaemoglobin within 44 hours, but no abnormal pigments

TABLE II.—*Incubation of Oxalated Human Whole Blood* (37° *C.*).

Time.	System.	pH.	Methaemoglobin. Neutral.	Methaemoglobin. Alkaline.	Methaem-albumin.	Schumm's test.
Before	Corpuscles	7·56	0	0	0	0
	Plasma	7·86	0	0	0	0
24 hours	Corpuscles	7·5	0	0	0	0
	Plasma	7·98	0	0	0	0
44 ,,	Corpuscles	7·4	0	0	0	0
	Plasma	7·9	0	0	0	0
67 ,,	Corpuscles	7·3	0	0	0	0
	Plasma	8·8	0	0	0	0
137 ,,	Corpuscles	7·3	+	0	0	0
	Plasma	8·9	+	+	+	++
161 ,,	Corpuscles	7·3	+	0	0	0
	Plasma	8·7	0	±	++	++
185 ,,	Corpuscles	7·47	+	0	0	0
	Plasma	8·7	0	±	++	++

++ = much present or strongly positive; + = positive or present; ± = trace; 0 = nil or negative.

were noted in either the corpuscles or plasma until 137 hours' incubation. Then the corpuscles on lysis were found to yield both haemoglobin and methaemoglobin (pH = 7·3), while the plasma (pH = 8·9) was found to contain haemoglobin, alkaline and neutral methaemoglobin and methaemalbumin. Schumm's test was positive for the first time. At 161 and 185 hours the corpuscles contained neutral methaemoglobin, while the plasma contained a trace of alkaline methaemoglobin, no neutral methaemoglobin and much methaemalbumin. Schumm's test was strongly positive.

These and other experiments indicated that within the corpuscles some of the oxyhaemoglobin had been converted into reduced haemoglobin, which was subsequently oxidized to methaemoglobin. As the pH of the lysed corpuscles had fluctuated between 7·56 to 7·3 during the experiment, there was naturally no tendency for the formation of alkaline methaemoglobin within the corpuscles. Once haemolysis had occurred and the corpuscular pigments diffused into the plasma, conditions were established which permitted the extracorpuscular production of alkaline methaemoglobin and methaemalbumin.

IV. OXYGEN REQUIREMENTS AND TEMPERATURE CONSIDERATIONS.

Neill (1925) recorded that oxygen was necessary for the spontaneous formation of methaemoglobin in blood or in haemoglobin solutions either at ordinary temperature or at 55° C., and that the temperature at which the system was incubated determined the rate of conversion. Solutions of haemoglobin and not whole blood were used throughout these experiments.

The work of Conant (1923), Conant and Fieser (1924) and Conant and Scott (1926) showed that it was necessary to have reduced haemoglobin in the presence of some oxidizing agent for methaemoglobin to be produced, and, as

emphasized by Peters and Van Slyke (1931), the conditions favouring the spontaneous production of methaemoglobin in haemoglobin solutions are those in which the haemoglobin is partly oxidized and partly reduced. During the present investigation it was found that oxygen was essential for the formation of methaemalbumin from haemoglobin.

(1) *Incubation of Reduced Haemoglobin Without Access to Oxygen.*

For purposes of the experiment the usual human plasma-haemoglobin mixture was prepared, and the oxyhaemoglobin subsequently was converted into reduced haemoglobin by treatment with excess of sodium hydrosulphite. It was then sealed in glass capsules so as to prevent access to air. Methaemoglobin and methaemalbumin both failed to appear even after incubation for 11 days at 37° C.

(2) *Changes in Haemoglobin at Room Temperature.*

It was found that when a sterile haemoglobin solution or a human plasma-haemoglobin mixture was kept at room temperature (18° to 22° C. in this experiment) in tubes plugged with cotton-wool, neither methaemoglobin nor methaemalbumin had appeared in demonstrable quantity after a week or longer. In one experiment, when a sterile haemoglobin solution with a pH = 7·4 was kept at 16° to 18° C. for 17 days, no methaemoglobin was demonstrable and the pH had meanwhile only changed to 8·0.

In sterile oxalated human blood stored under similar conditions for two weeks no methaemoglobin was found in the lysed corpuscles, while the plasma contained haemoglobin derived from spontaneous lysis of the corpuscles, but no methaemoglobin or methaemalbumin.

It was then decided to ascertain whether a human plasma-methaemoglobin system would yield methaemalbumin at room and body temperatures.

V. INCUBATION OF METHAEMOGLOBIN AND HUMAN PLASMA.

For this purpose, solutions of methaemoglobin prepared from haemoglobin by the addition of potassium ferricyanide were incubated at 37° C. in the presence of human plasma. By the second day methaemalbumin was found to have replaced the original methaemoglobin, differing from it both spectroscopically and in its chemical behaviour to Stokes's reagent, dilute ammonium sulphide (10 per cent.) and strong ammonium sulphide. On repeating the experiment, methaemalbumin was demonstrated within seven hours.

When methaemoglobin solutions similarly prepared were kept at 16° to 18° C. in the presence of human plasma, methaemalbumin was definitely detected within 44 hours.

The result of a typical experiment conducted at 16° to 18° C. may be studied in Table III. It will be noted that the pH changed from 7·8 to 9·0 and that alkaline methaemoglobin and a trace of methaemalbumin appeared in 24 hours. By 44 hours methaemalbumin was demonstrable spectroscopically and a strongly positive Schumm's test was recorded. In a solution of methaemoglobin used as a control at this temperature the pH changed from 7·2 to 7·0 after 141 hours and no other pigments developed.

TABLE III.—*Neutral Methaemoglobin and Human Plasma at Room Temperature (16°–18° C.).*

Time in hours.	System.	pH.	Alkaline methaemoglobin.	Methaem-albumin.	Schumm's test.
Before	Plasma + Met. Hb	7·8	0	0	0
	Methaemoglobin	7·2	0	0	0
24	Plasma + Met. Hb	..	..	±	±
	Methaemoglobin	7·2	0	0	0
44	Plasma + Met. Hb	8·7	+	+	++
	Methaemoglobin	..	0	0	0
69	Plasma + Met. Hb	..	+	+	++
	Methaemoglobin	..	0	0	0
141	Plasma + Met. Hb	9·0	+	+	++
	Methaemoglobin	7·0	0	0	0

Met. Hb. = methaemoglobin; ++ = much present or strongly positive; + = present or positive; ± = trace present; 0 = nil or negative.

VI. SULPHAEMOGLOBIN AND HUMAN PLASMA (37° C. AND 40° C.).

Sulphaemoglobin was prepared by bubbling H_2S through a solution of oxyhaemoglobin derived from well-washed, lysed, human corpuscles. One volume of this was incubated at 40° C. with 4 volumes of human plasma. By the third day all sulphaemoglobin had been replaced by methaemalbumin. The same result was obtained when a human plasma-sulphaemoglobin system was incubated at 37° C.

Incubation experiments were also made with sulphaemoglobin derived from the corpuscles of a patient suffering from sulphanilamide poisoning. No sulphaemoglobin was found in the plasma, and the condition was really one of sulphaemoglobincythaemia rather than of sulphaemoglobinaemia as generally stated. The corpuscles were laked with 4 volumes of distilled water and a well-defined α band in the red portion of the spectrum was found at 618$\mu\mu$; on a Hartridge reversion spectroscope this band was colinear with that of sulphaemoglobin prepared chemically from haemoglobin. The α band was not dispersed by ammonium sulphide (10 per cent.), sodium hydrosulphide or sodium cyanide, though it was discharged by hydrogen peroxide (10 " volumes "). When this mixture of oxyhaemoglobin and sulphaemoglobin was incubated at 40° C. in the presence of human plasma, it was found on the third day that the sulphaemoglobin had disappeared and been replaced by methaemalbumin. Thus the α band in the red portion of the spectrum at 623$\mu\mu$ was colinear with the α band of methaemalbumin found in blackwater fever plasma, and was not dispersed by H_2O_2 (10 volumes) or 10 per cent. ammonium sulphide though it was discharged by sodium hydrosulphite.

When the sulphaemoglobin plasma system was kept at 18°–22° C. methaemalbumin had not appeared by the fourth day.

VII. INCUBATION OF MONKEY PLASMA AND HAEMOGLOBIN (37° C. AND 40° C.).

Experiments at 37° and 40° C. were carried out with plasma derived from different species of monkeys (*Macacus rhesus*, *M. sinicus* and *Cercopithicus aethiops*) and simian haemoglobin. The results were similar to those observed

when human plasma-haemoglobin mixtures were incubated. Data from a typical experiment are summarized in Table IV.

TABLE IV.—*Incubation of Monkey Plasma* (Cercopithicus aethiops) and *Haemoglobin* (40° *C.*).

Time.	System.	pH.	Methaemoglobin.		Methaem-albumin.	Schumm's test.
			Neutral.	Alkaline.		
Before	Haemoglobin	7·6	0	0	0	0
	Plasma + Hb	8·0	0	0	0	0
19 hours	Haemoglobin	7·5	+	0	0	0
	Plasma + Hb	9·0	0	±	±	±
43 ,,	Haemoglobin	7·6	+	0	0	0
	Plasma + Hb	9·2	0	+	+	+
67 ,,	Haemoglobin	7·5	+	0	0	0
	Plasma + Hb	..	0	0	++	++
91 ,,	Haemoglobin	7·6	+	0	0	0
	Plasma + Hb	9·2	0	0	++	++
115 ,,	Haemoglobin	7·6	+	0	0	0
	Plasma + Hb	9·2	0	0	++	++

Final haemoglobin concentration = 10 per cent. (Haldane). ++ = much present or strongly positive; + = present or positive; ± = trace present; 0 = nil or negative; Hb = haemoglobin.

It will be noted that when the control system, consisting of an aqueous solution of haemoglobin, was incubated at 40° C. no significant changes were observed in the pH, which varied from 7·5 to 7·6; neutral methaemoglobin appeared in 19 hours and was demonstrable throughout the experiment.

In the plasma-haemoglobin system there was a change in pH from 8·0 to 9·2. At 43 hours alkaline methaemoglobin and methaemalbumin were found, but from 67 hours onwards only methaemalbumin was demonstrable. Schumm's test was definitely positive from 43 hours onwards.

VIII. INCUBATION OF RABBIT PLASMA AND HAEMOGLOBIN.

Similar experiments were undertaken with rabbit plasma-haemoglobin mixtures incubated at 37° and 40° C. The results of a typical experiment are epitomized in Table V.

TABLE V.—*Incubation of Rabbit Plasma and Haemoglobin* (40° *C.*).

Time in hours.	pH.	Methaemoglobin.		Methaem-albumin.	Schumm's test.	Haematin-like spectrum.
		Neutral.	Alkaline.			
Before	7·8	0	0	0	0	0
24	8·3	+	+	0	0	0
44½	8·6	0	+	0	0	0
67½	..	0	+	0	+	+
91½	8·7	0	+	0	++	+

Final haemoglobin concentration = 12 per cent.; ++ = much present or strongly positive; + = present or positive; ± = trace present; 0 = nil or negative.

As with human and simian plasma-haemoglobin systems, there was a shift of pH to the alkaline side of 8·0. Within 24 hours neutral methaemoglobin was evident, the α band at 630μμ being dispersed by dilute ammonium sulphide (10 per cent.). By 44½ hours this band in the red had disappeared, but on adding *N* HCl (1 drop to 2 c.c.) it reappeared at 630μμ. By 67½ hours there was a broad band at 616μμ resembling alkaline haematin. Schumm's test was positive. On adding *N* HCl (1 drop to 2 c.c.) an α band in the red at 629μμ appeared which was discharged by 10 per cent. ammonium sulphide. At 91 hours a spectrum resembling alkaline haematin was still present and Schumm's test was strongly positive. Neutral methaemoglobin again appeared on the addition of *N* HCl, showing that alkaline methaemoglobin was present. Methaemalbumin, however, was never demonstrable.

Similar results were obtained in other experiments using plasma or sera derived from a number of other mammals, including the guinea-pig, rat, sheep, goat, horse and ox; methaemalbumin was never demonstrated with any of these animal sera.

DISCUSSION.

It will be noted that conditions such as deprivation of oxygen or storage at 16° to 18° C., which prevented the spontaneous formation of methaemoglobin from haemoglobin, also precluded the formation of both methaemoglobin and methaemalbumin in a plasma-haemoglobin mixture.

When solutions of oxyhaemoglobin were incubated at 37° or 40° C., the pH showed no alkaline shift and neutral methaemoglobin constantly appeared. In plasma-haemoglobin mixtures there was a shift to the alkaline side of 8·0, and both neutral and alkaline methaemoglobin were demonstrated soon after incubation and before methaemalbumin had appeared. These findings suggested that methaemoglobin represented an intermediary stage in the breakdown of haemoglobin to haematin and methaemalbumin. The subsequent demonstration of haematin or methaemalbumin appeared to depend on the species of mammal from which the serum or plasma was derived, the source of haemoglobin being immaterial. Thus it was repeatedly found that haemoglobin derived from any species of mammal would give rise to methaemalbumin if incubated at 37° or 40° C. in the presence of human or simian plasma or serum.

The mammalian plasma tested fell into two groups: (1) The human or simian group in which a pigment with an α band at 623μμ to 624μμ formed on incubating haemoglobin in the presence of plasma at 37° or 40° C.; and (2) the rabbit group, which included all the species of mammalian plasma tested other than that of men and monkeys, in which a spectrum resembling alkaline haematin with an α band about 616μμ was produced.

The interpretation originally placed on these findings was that there were two recognizable stages in the spontaneous disintegration of haemoglobin in the presence of rabbit plasma, and three in the presence of human and simian plasma.

(1) In the first stage, part of the oxyhaemoglobin was converted to reduced haemoglobin which underwent spontaneous oxidation to neutral methaemoglobin; owing to the shift in the pH of the haemoglobin-plasma systems to

the alkaline side of 8·0, this neutral methaemoglobin tended to be converted into alkaline methaemoglobin.

(2) The splitting of methaemoglobin into globin and haematin (ferric).

(3) The coupling of haematin (ferric) with some plasma protein to form the pigment characterized by a constant α band at 623$\mu\mu$ to 624$\mu\mu$.

This latter hypothesis was later confirmed when it was found *in vitro* that an identical pigment was immediately formed when alkaline haematin (ferric) derived from purified haemin was added to (1) human or simian plasma, (2) the albumin fraction of human or simian serum, and (3) to human crystalbumin but not to the other proteins, such as globoglycoid or seroglycoid, which were also contained in the albumin fraction. It was on the basis of these findings that the pigment was re-named methaemalbumin.

The conclusion reached in regard to the mammalian plasma of rabbit type was that haematin failed to couple with the serum-albumin which remained free.

More recent investigations on the behaviour of rabbit albumin-haematin mixtures in the ultracentrifuge undertaken by Dr. A. S. McFarlane indicate, however, that rabbit serum albumin does combine with haematin (ferric), though the haematin-albumin compound formed differs spectroscopically and in certain other respects from simian and human methaemalbumin.

The Catabolism of Extracorpuscular Circulating Hæmoglobin in Man.

In intravascular haemolysis of any magnitude in man some of the free circulating haemoglobin is excreted in the urine as such, and more of it is absorbed by the R.E. system with the production of bilirubin and the iron-containing pigment, haemosiderin. The remainder, which persists in the circulation, gives rise to methaemalbuminaemia—the so-called " haematinaemia " of previous writers.

The bearing of these *in vitro* experiments on the mode of disintegration of residual circulating haemoglobin in the haemoglobinurias and certain types of haemolytic anaemia will receive consideration in other publications. It may, however, be mentioned here that to date the writer has failed to demonstrate extracorpuscular methaemoglobin as a precursor of methaemalbumin in the plasma in the intravascular haemolysis of man. Another route may therefore be followed in the circulating blood, namely, (1) the splitting of haemoglobin into globin and reduced haematin (ferrous); (2) the oxidation of reduced haematin (ferrous) to haematin (ferric); (3) the coupling of haematin (ferric) with serum albumin to form methaemalbumin. In this regard it is noteworthy that when reduced haematin (ferrous) is injected into the cardiac cavity of monkeys, methaemalbumin is immediately formed.

SUMMARY AND CONCLUSIONS.

(1) When a haemoglobin-plasma system is incubated (37° or 40° C.) neutral methaemoglobin is produced in the early stages, which is liable to be converted into alkaline methaemoglobin owing to a shift in pH of plasma to the alkaline side of 8·0.

(2) When an aqueous solution of haemoglobin is incubated alone this alkaline shift is absent and only neutral methaemoglobin is demonstrable.

(3) Where oxalated whole blood is incubated (37° or 40° C.) no alkaline shift in the pH is observed in the sedimented corpuscles which ultimately contain neutral methaemoglobin ; in the plasma, owing to the alkaline shift, alkaline methaemoglobin is formed.

(4) If the plasma in the system be derived from man and monkeys a pigment —methaemalbumin—with an α band at 623$\mu\mu$ to 624$\mu\mu$ appears within 48 hours and increases progressively—apparently at the expense of methaemoglobin.

(5) After a period of incubation at 37° C. for 10 days methaemalbumin is present in considerable concentration ; it can be isolated in the albumin fraction entirely free from haemoglobin or methaemoglobin ; on drying *in vacuo* over sulphuric acid a brown solid residue is formed containing methaemalbumin which is water-soluble and present in a stable and relatively pure form.

(6) The conclusion reached is that *in vitro* there are three stages demonstrable in the katabolism of haemoglobin incubated in the presence of human or simian plasma : (1) The production of neutral and alkaline methaemoglobin, (2) the splitting of methaemoglobin into globin and haematin (ferric), and (3) the coupling of haematin (ferric) to serum albumin to form methaemalbumin.

(7) With rabbit plasma and that of many other mammals the pigment produced in the later stages of incubation resembles haematin (ferric) spectroscopically, but there is some evidence that even here a haematin-albumin compound is formed.

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